Types of Resistance and Compatibility in Slash Pine Seedlings Infected by Cronartium fusiforme

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ABSTRACT

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Seedlings of slash pine were inoculated with basidiospores of *Cronartium fusiforme* and examined periodically to determine symptoms and host responses to infection. Histological examination of inoculated seedlings provided evidence of four general types of host responses: (i) no penetration or infection; (ii) subliminal infection; (iii) three related types of hypersensitivity; and (iv) typical gall development. The hypersensitive responses effectively confined the fungus to reaction zones of necrotic cells.

Additional key words: fusiform rust.

Fusiform rust of southern pines, which is caused by *Cronartium fusiforme* Hedgc. & Hunt ex Cumm., is the most destructive disease of the southern pines. A recent estimate has placed annual losses from this disease at $28 million (8), and there is evidence that the incidence of the disease is increasing (9). These losses, plus the increasing costs involved in intensive forest management emphasize the need to find pines with resistance to fusiform rust.

A rapid and reproducible method has been developed for inoculating large numbers of pine seedlings with known concentrations of basidiospores of *Cronartium fusiforme* (5). This procedure, known as the concentrated basidiospore system, yields estimates of resistance to fusiform rust, but it provides no information as to why progeny from certain parent trees are more resistant than others.

During the development of the concentrated basidiospore system, thousands of slash pine (*Pinus elliottii* Engelm. var. *elliottii*) seedlings from about 100 half-sib families and from mixed seedlots periodically were examined in detail to determine the types of symptoms and host-parasite interactions that occur in seedlings with degrees of resistance to *C. fusiforme*. To obtain basic information on the differences between resistant and susceptible slash pines, a histological study was undertaken with samples of the inoculated seedlings.

MATERIALS AND METHODS

Pine culture and inoculation.—Seeds of slash pine (half-sib families) were planted in flats containing a soil, pine bark, and sand mixture (2:1:1, v/v) and maintained in a greenhouse. When the hypocotyls were about 2.5 cm long, seedlings were transplanted into plastic flats (33 × 13 × 11 cm) that contained the same soil mixture as the germination flats. Twenty or 30 seedlings were planted in each plastic flat in two or three rows of 10 seedlings. Seedlings were inoculated 4 or 6 weeks after transplanting with a water suspension of basidiospores applied either with a pressurized sprayer designed for applying indicator reagents to chromatograms or by passing the flats under a spray nozzle on a conveyor belt. The concentration of basidiospores (7.5×10^4/ml) was determined with a Coulter electronic particle counter. Inoculated seedlings were placed in a mist chamber at 20 C for 24 hours, held on a laboratory bench for an additional 24 hours, then moved to a greenhouse bench.

The second phase of this research involved seedlings grown under different cultural conditions and inoculated by a different technique. Seeds of slash pine from a seedlot of unknown resistance were planted in flats that contained sand and peat moss mixture (1:1, v/v) and maintained in a greenhouse. Seedlings were transplanted into pots (10-cm) that contained a mixture of soil, sand, and peat moss (2:1:1, v/v) with two seedlings per pot. At ages ranging from 17 days to 4.5 months, seedlings were inoculated either on the hypocotyls or at one of two positions on the stems. The exact points of inoculation and the technique, involving transfer of precast basidiospores from Millipore filters to the seedlings, have been described previously (6). Inoculated seedlings were placed in a mist chamber at 20 C for 54 hours then moved

to a greenhouse with supplemental light to provide 14 hours of continuous light every 24 hours.

Histological procedures.—A flat of 20 or 30 seedlings of each half-sib family inoculated with a suspension of basidiospores was chosen at random for detailed observations and histological examination. Every seedling in each sample flat was examined at 2, 4, and 6 weeks after inoculation for symptoms of infection by *C. fusiforme* (yellow, red, or purple lesions on the stems and needles). Additional examinations were made at 3, 6, 9, and 12 months after inoculation. Each stem spot and each needle infection that had progressed into the stem, based on color development around the site of infection, was recorded and described as to time of appearance, morphological characteristics, and position in reference to the cotyledonary node. Selected seedlings with different symptoms were collected at intervals for histological examination. These specimens were processed by standard histological methods (4) and stained with Pianeze III-B stain (11). The dewaxed sections were placed in saturated chloral hydrate in 95% ethyl alcohol for 30 minutes and rinsed for 5 minutes in water just prior to staining. Sections were stained for 30 minutes.

Seedlings inoculated at specific sites were collected weekly through 8 weeks after inoculation. These specimens were processed as above except that the sections were stained with periodic acid-Schiff reagent (1).

RESULTS AND DISCUSSION

Four general types of host-parasite interactions were observed.

Type 1—No apparent penetration or infection.—Basidiospores germinated on the epidermis at about the same estimated frequency as on seedlings that became infected, but no penetrations were observed even though many of the germs tubes had produced appressoria.

There are two possible explanations for the type 1 response: (i) Since *C. fusiforme* infects only the succulent, relatively immature portion of stems and primary needles of pine seedlings (7), the failure of basidiospores to penetrate these seedlings may have been due to the maturity of the organs at the time of inoculation. (ii) Since appressoria were formed by germ tubes of some basidiospores, incipient penetration may have occurred

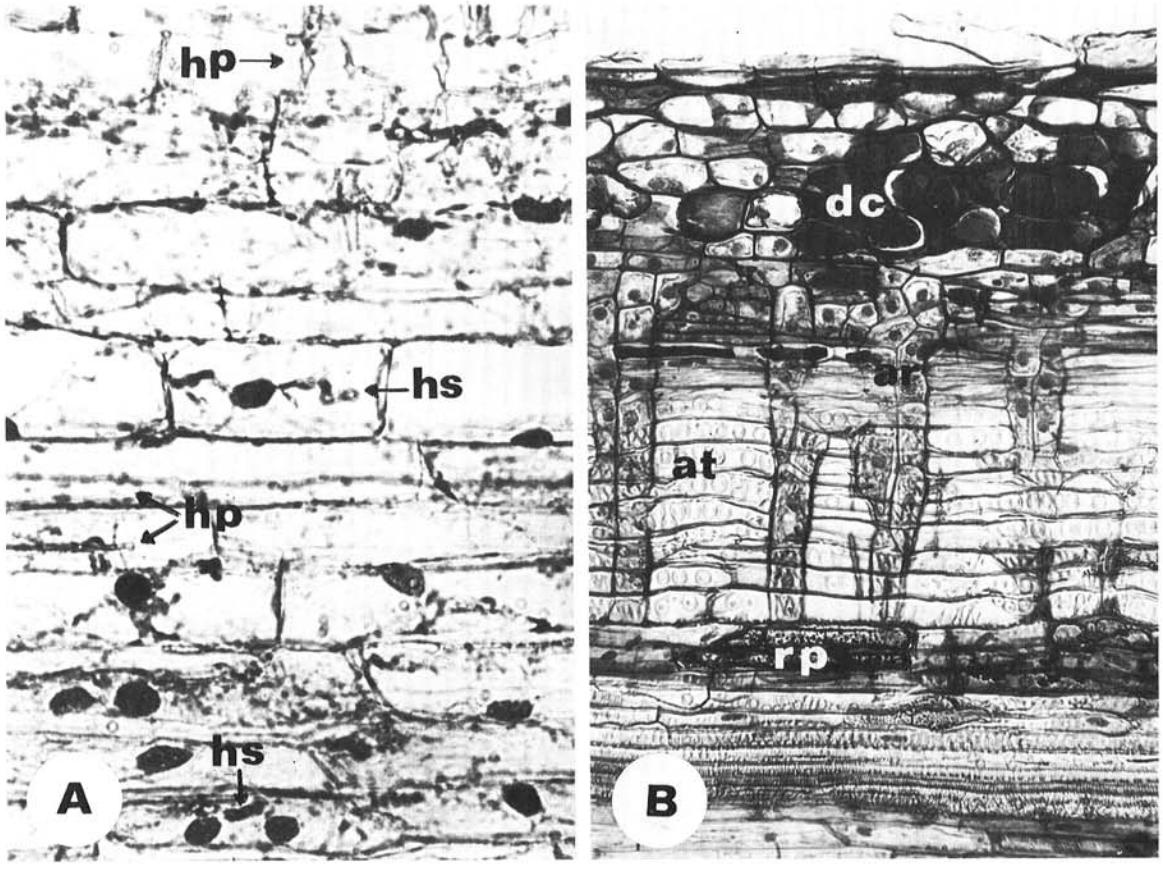

Fig. 1-(A, B). Type 2 and type 4 host responses of slash pine seedlings to *Cronartium fusiforme*. **A)** Typical type 2 response in cortex of infected slash pine seedling 8 weeks after infection showing no distortion of cortical cells and only scattered hyphae (hp) and haustoria (hs) (×500); and **B)** typical type 4 response of infected slash pine seedling 8 weeks after infection showing distorted cortical cells (dc), abnormal rays (ar) and tracheids (at), and reaction parenchyma (rp) (×125).

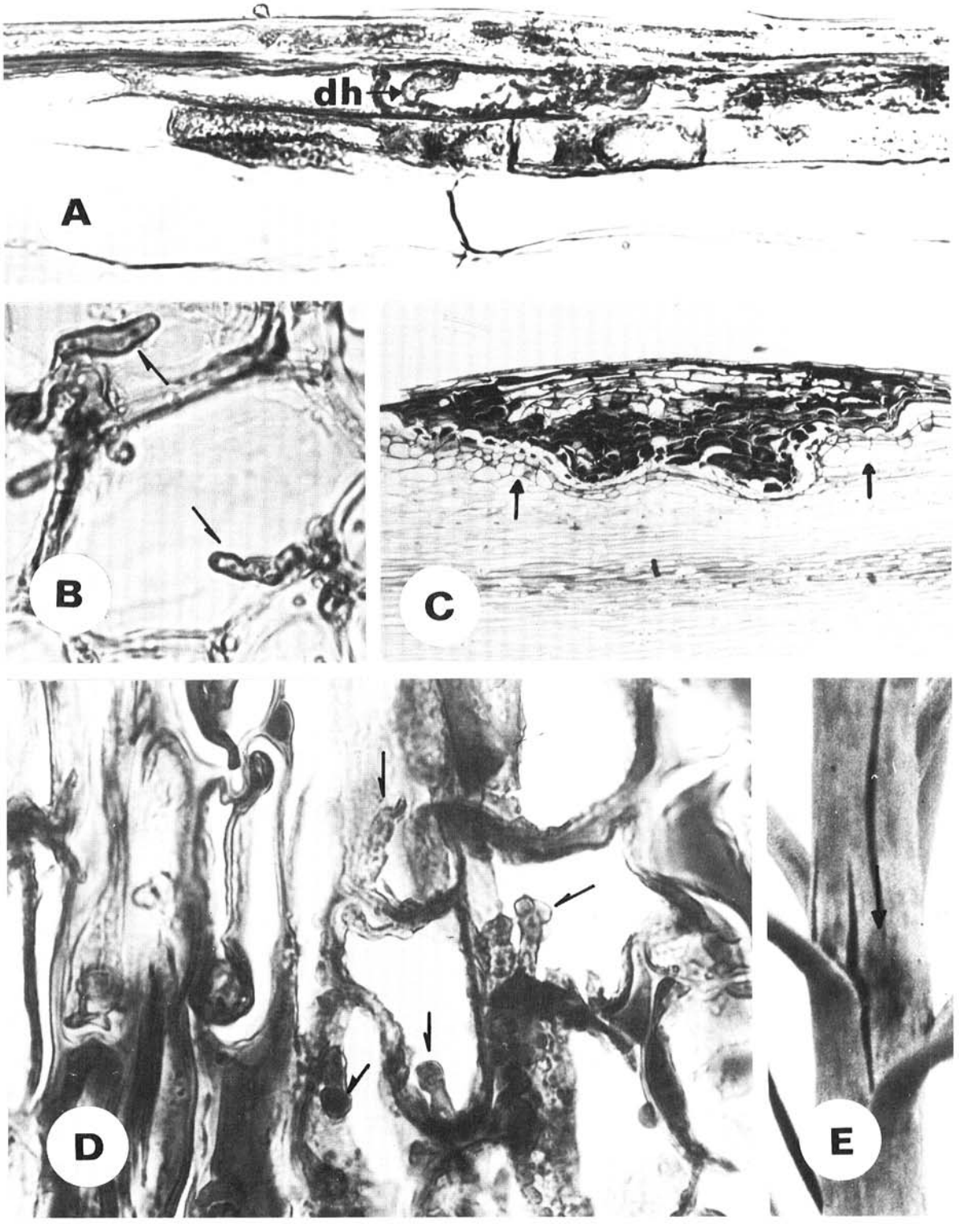

Fig. 2-(A to E). Type 3a and 3b host responses of slash pine seedlings to *Cronartium fusiforme*. **A)** Longitudinal section through a typical type 3a response 2 weeks after infection showing limits of colonization by the fungus and the encrusted, distorted haustoria (dh) (×500); **B)** normal haustoria (arrows) (×500); **C)** longitudinal section through a typical type 3b response 6 weeks after infection showing the reaction zone of necrotic cells and the layer of cells (arrows) between the reaction zone and nonaffected cells (×35); **D)** an internal portion of a type 3b reaction zone showing distortion of affected cortical cells with deposits and distorted haustoria (arrows) (×500); and **E)** typical lesion on a slash pine stem which is indicative of a type 3b host response (×6).

without being detected by our techniques. Thus, a hypersensitive response may have occurred at the cellular level. Such a response would be a useful and important mechanism of resistance. The possibility of such a mechanism is supported by the observation that a number of seedlings in certain resistant families of slash pine did not develop observable symptoms following inoculation.

Type 2–Subliminal infection.—This type of host response to infection occurred less frequently than any other. The epidermal and cortical cells at the point of infection were distinguishable from the surrounding, nonaffected cells only by a slight increase in the intensity of staining. There was little distortion of host cells and no evidence of gall development 8 weeks after inoculation. Mycelium was very sparse in the infected areas, and haustoria were few and scattered (Fig. 1-A, 1-B). However, the pattern and rate of colonization and the volume of stem tissues colonized were about the same as that observed in seedlings undergoing typical gall development (7).

The failure of the parasite to induce a greater response in these seedlings, even after reaching the cambium, suggests the existence of slash pines that are inherently physiologically less reactive to the pathogen, or that virulence in *C. fusiforme* is variable. Since there is evidence of variability in different isolates of *C. fusiforme* (10), it is reasonable to speculate that a type 2 response involved an avirulent isolate of the fungus.

The type 2 response also may have been latent infections that would eventually become typical galls. Latent infections could account for seedlings that are graded as healthy at nurseries but later develop galls at positions on the stems that could have resulted only from infections in the nursery beds. Unfortunately, the frequency of this response can only be estimated by sectioning the seedlings since there are no macroscopic symptoms.

Type 3–Hypersensitive reactions.—This host response produced three general types of reaction zones which are designated 3a, 3b, and 3c.

Type 3a–Superficial corticular hypersensitivity.—In this response a localized, darkly-stained reaction zone developed on the stem at and immediately around the point of infection and was composed of necrotic cells to which the fungus was effectively confined. Histological examination 2 weeks after inoculation showed that the reaction zones were two to three cells deep into the stem cortex and three to five cells wide (Fig. 2-A). Although their viability was not tested, the heavily stained host cells and the fungal structures in these areas appeared to be dead. The few haustoria that developed in these reaction zones were encrusted, distorted, and appeared distinctly granular in contrast to haustoria formed in a susceptible host response (Fig. 2-B).

Type 3b–Corticular hypersensitivity.—This host response was similar to type 3a but differed in size, rate of development, and in differentiation of cells between the reaction zone and nonaffected cells. In longitudinal section, the lesions were semicircular to approximately rectangular areas of necrotic cells confined to the outer cortical cells of the stem (Fig. 2-C). The reaction zones were delineated from the nonaffected cortical cells by a layer of globose-to-rectangular cells with thin walls that stained more intensely than the cortical cells but less intensely than the cells within the reaction zone. More mycelium and haustoria occurred in the reaction zones of type 3b than type 3a but they had the same general degenerate appearance.

Cells within the reaction zones were distorted and either empty or filled with small granules or larger oil-like globules. When Pianeze III-B stain was used, most of the cells in the reaction zone stained an intense green; a few scattered cells were yellow or dark purple. Cells with inclusions stained light green or yellowish-brown. Hyphae and haustoria within the reaction zone stained either dark purple or dark green. Fungal cells that stained purple were only slightly distorted and encrusted; those that stained green were heavily encrusted and distorted and had granular or globose inclusions such as those in the host cells (Fig. 2-D). Apparently, this hypersensitive-like response occurred in advance of the cells colonized because the fungus was found no closer than several cells back from the border of the reaction zone. The pathogen was effectively confined to the zone in type 3b reactions, and disease development was completely stopped with only superficial damage to the host.

In both types 3a and 3b purple stem lesions were visible to the unaided eye as early as 2 weeks after inoculation. Type 3a lesions reached their maximum size at this time whereas the 3b type lesions continued to develop for up to 3 months after inoculation. After 3 months, these lesions ranged in size from about 0.4-2.0 × 1.0-4.0 mm and varied in shape from approximately circular to elliptical or somewhat rectangular (Fig. 2-E). The long axes of the lesions generally were aligned longitudinally.

Type 3c–Stabilized galls.—This type of host response also confined the fungus to a reaction zone of necrotic cells but was characterized by different morphological symptoms and involved other host tissues in addition to the cells of the cortex.

Examination of inoculated seedlings revealed some with small galls that had stopped enlarging after 3 months. A typical gall 1 year after inoculation was 30-60 mm long, and the diameter was 1.5 to 2 times that of the stem below the gall. Stabilized galls stopped enlarging after 3 months, seldom exceeded 8 mm in length, and were only 1 to 3 mm larger in diameter than the stem below the gall.

No colored stem lesions developed on the stabilized galls. In all cases, however, data from earlier examinations indicated that an infected primary needle had been present in the area of the gall and that the fungus had grown from the needle into the stem. Needle infections were readily recognizable by the characteristic yellow, red, or purple areas that developed at and around the point of infection in primary needles. When the discolored area extended to the stem, the fungus had spread through the needle into the stem (7). Histological examination of stabilized galls revealed (i) that the fungus had become established in the stems only from the infected needles, and (ii) that internal reaction zones developed in the stems only after the fungus entered the stem tissues from the needle trace.

Seedlings with symptoms indicating that the fungus had spread through infected needles into the stems were examined histologically 3 months after inoculation. Varying responses were observed. In some seedlings the fungus had spread through the needle and needle trace to

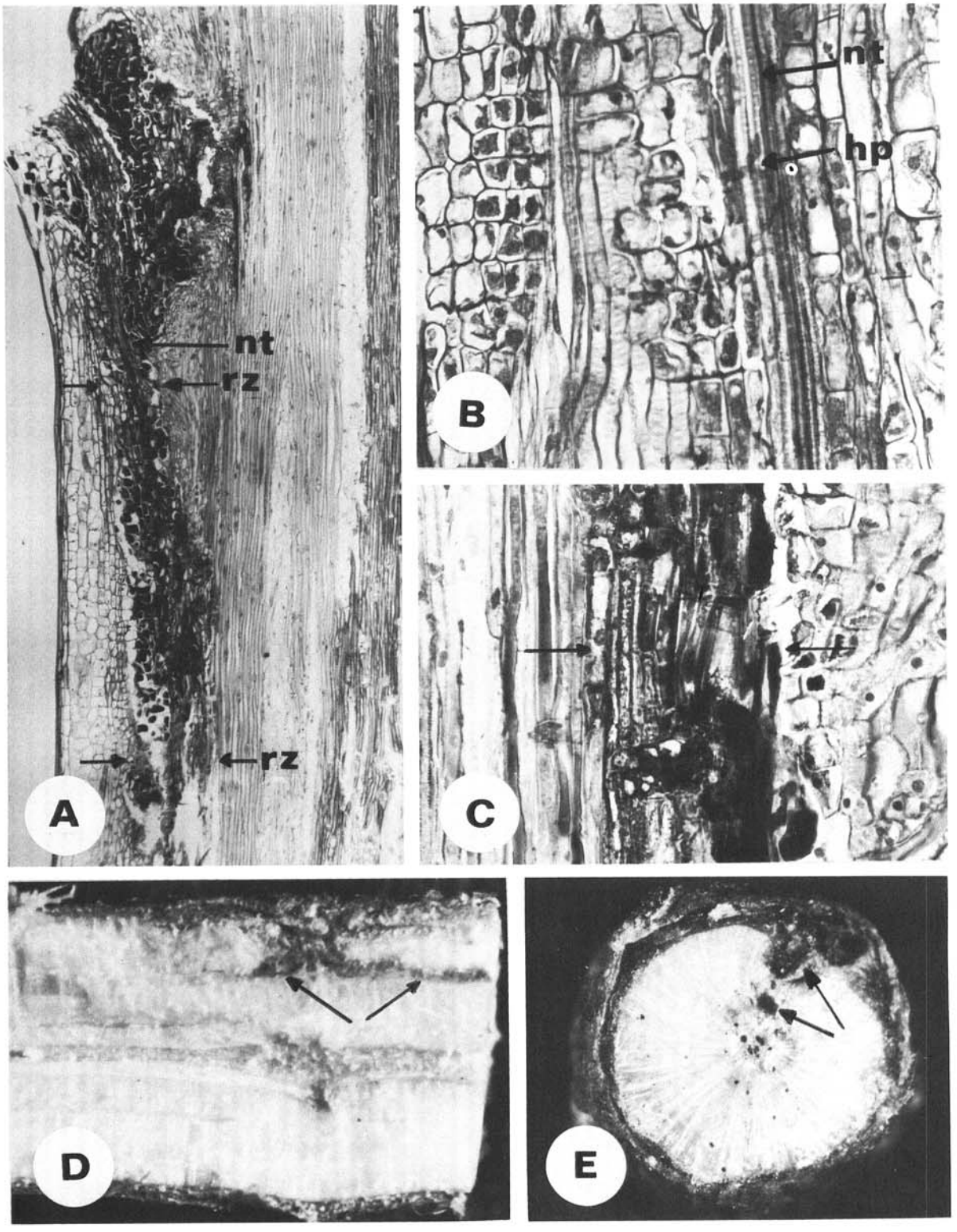

Fig. 3-(A to E). **A)** A longitudinal section of a typical type 3c host response in slash pine seedlings 3 months after infection by *Cronartium fusiforme* showing an infected needle trace (nt) and a reaction zone (rz) between tracheids to the inside and cortical cells to the outside (×35); **B)** longitudinal section through an infected stem 3 months after infection showing the fungus (hp) growing into the stem through the needle trace (nt) with no observable host response (×125); **C)** a portion of a typical type 3c reaction zone (arrows) showing necrotic, abnormal tracheids and ray cells (×125); and **D**, and **E)** longitudinal and transverse sections of slash pine seedlings 12 months after infection showing the reaction zones (arrows) produced by type 3c host responses (×6).

the vascular cambium and typical gall development followed (Fig. 3-B). In contrast, seedlings with the type 3c response showed a dramatic reaction to the pathogen (Fig. 3-A). In these seedlings, the fungus grew through the needle trace and apparently reached the cambium before the major host response occurred. This conclusion is based on the fact that the innermost portion of the reaction zone extended longitudinally along the cambial area for some distance and two to four strands of infected, modified xylem delineated the inner margin of the zone. At 3 months after inoculation, the typical type 3c reaction zone, in longitudinal section, consisted of a zone of darkly stained, necrotic cortical cells surrounding all or part of the affected needle trace from where the needle emerged from the stem to the vascular tissues of the stem. The reaction zone then followed the needle trace and extended longitudinally along the area where the vascular cambium had been at the time the fungus had spread to this point. This innermost portion of the reaction zone extended, mostly downward, from the end of the needle trace and consisted of darkly stained and drastically altered xylem and ray cells (Fig. 3-C). Neither hyphae nor haustoria were observed in any host cells beyond the margins of the type 3c reaction zones.

To confirm that the type 3c response confines the pathogen to the reaction zone, seedlings with galls that had not enlarged between 3 and 12 months after inoculation were examined. These seedlings were sectioned longitudinally or transversely through the centers of the galls. Examination of these stabilized galls confirmed that (i) the fungus had entered the stems only from infected needle traces, (ii) the type 3c host response was responsible for the stabilized galls, (iii) the fungus was effectively confined to the reaction zones, and, (iv) the seedlings were "recovering" from the infection; i.e., the cambium was reforming around the reaction zones (Fig. 3-D, 3-E).

The type 3c response is a type of resistance in the progeny of some slash pine selections. It is interesting that there was no evidence of resistance in needle tissues. The type 3c response seemed to occur only after the fungus reached the cambial area and spread for some distance longitudinally. At this time, there was a host response in which a reaction zone developed from the infected, modified xylem along the infected needle trace to the point where the needle entered the stem. This is an important resistant response that must be considered in screening slash pine seedlings for resistance to *C. fusiforme*. It is essential to permit sufficient time between inoculation and final evaluation for the accurate recognition of the stabilized galls resulting from this type reaction.

The type 3c host response may have been responsible for the "recovery" of galled slash pine seedlings reported by Jewell and Snow (2). The appearance of an isolated zone of necrotic tissues that they reported is quite similar to the type 3c response we report here.

The type 3 host responses observed on inoculated seedlings of slash pine all confined the pathogen to a zone of necrotic cells. While these responses may be observed on any group of seedlings that are inoculated, they occurred far more frequently in the progeny of selected slash pines that were judged resistant based on percentages of seedlings with galls 9-12 months after inoculation. Similar morphological symptoms often developed on the progeny of susceptible slash pines, but in these seedlings the fungus generally escaped the reaction zone and continued to parasitize the seedlings. In these instances, the difference between resistance and susceptibility appeared to be more quantitative than qualitative. Resistant seedlings apparently respond and accumulate inhibitory or toxic substances in the reaction zones more rapidly, and perhaps in greater quantities, than the susceptible seedlings. Additional research is presently underway to determine the physiologic nature of the type 3 responses in the resistant seedlings which prevents development of the fungus beyond the reaction zone.

Type 4–Normal gall development.—Infection and host-parasite interaction culminate in the production of a gall. The patterns of colonization by the fungus, tissues colonized, and alteration in host tissues have been described (3, 7). The sequence of host responses that occurred through 8 weeks were (i) development of a small, darkly-stained reaction zone at the point of infection, (ii) spread of hyphae in all directions from the reaction zone with haustorial development some distance behind the margins of the actively spreading hyphal strands, (iii) obvious distortion of cortical cells after 3 weeks, and (iv) gall development starting between 3 and 5 weeks after inoculation (Fig. 1-B).

The four general types of slash pine responses to *C. fusiforme* that we have described represent an initial step in our efforts to understand the genetics and physiology of resistance in this host-parasite system. We identified a potential (type 1) and a definite (type 3) form of resistance. Additional study of the type 1 response may determine why basidiospores produce apparently normal germ tubes and appressoria on certain individuals but fail to infect. Types 3a, b, and c definitely are effective and useful mechanisms of resistance that operate consistently in progeny of certain slash pines. Slash pines that exhibit the type 3 response should receive maximum attention in breeding for resistance to fusiform rust.

The undisputed economic importance of fusiform rust of pine trees in the Southeastern USA makes it essential that the search for resistance be given a high priority in forestry research. The manipulation and effective deployment of host genes for resistance to fusiform rust will be required if genetic resistance is to become an effective tool for control of the disease.

LITERATURE CITED

1. FARRIS, S. H. 1966. A staining method for mycelium of Rhabdocline in Douglas-fir needles. Can. J. Bot. 44:1106-1107.
2. JEWELL, F. F., and G. A. SNOW. 1972. Anatomical resistance to gall-rust infections in slash pine. Plant Dis. Rep. 56:531-534.
3. JEWELL, F. F., R. P. TRUE, and S. L. MALLETT. 1962. Histology of Cronartium fusiforme in slash pine seedlings. Phytopathology 52:850-858.
4. JOHANSEN, D. A. 1940. Plant microtechnique. McGraw-Hill, New York. 523 p.
5. MATTHEWS, F. R., and S. J. ROWAN. 1972. An improved method for large-scale inoculations of pine and oak with Cronartium fusiforme. Plant Dis. Rep. 56:931-934.

6. MILLER, T. 1970. Inoculation of slash pine seedlings with stored basidiospores of Cronartium fusiforme. Phytopathology 60:1773-1774.
7. MILLER, T. 1972. Infection and colonization of slash pine seedlings by Cronartium fusiforme. Ph.D. Thesis. North Carolina State University, Raleigh. 91 p.
8. POWERS, H. R., JR., J. P. MC CLURE, H. A. KNIGHT, and G. F. DUTROW. 1974. Incidence and financial impact of fusiform rust in the South. J. For. 72:398-401.
9. SCHMIDT, R. A., R. E. GODDARD, and C. A. HOLLIS. 1974. Incidence and distribution of fusiform rust in slash pine plantations in Florida and Georgia. Fla. Agric. Exp. Stn. Tech. Bull. 763. 21 p.
10. SNOW, G. A., R. J. DINUS, and A. G. KAIS. 1975. Variation in pathogenicity of diverse sources of Cronartium fusiforme on selected slash pine families. Phytopathology 65:170-175.
11. VAUGHAN, R. E. 1914. A method for the differential staining of fungus and host cells. Ann. Mo. Bot. Gard. 1:241-242.