Infection and Colonization of Different Organs of Slash Pine Seedlings by Cronartium fusiforme

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Portion of a Ph. D. thesis submitted to the Graduate School, North Carolina State Univeristy, Raleigh, North Carolina.

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Cooperative investigations of the U. S. Department of Agriculture Forest Service and the North Carolina Agricultural Experiment Station, Raleigh. Journal Series Paper No. 4772 of the North Carolina Agricultural Experiment Station.

Accepted for publication 24 August 1976.

ABSTRACT

MILLER, T., and E. B. COWLING. 1977. Infection and colonization of different organs of slash pine seedlings by Cronartium fusiforme. Phytopathology 67: 179-186.

Slash pine seedlings ranging from 17 days to 4.5 mo old were inoculated with precast basidiospores of *Cronartium fusiforme* at predetermined locations on different organs to determine susceptibility of the organs at several ages and patterns of colonization by the fungus. Hypocotyls, stems, cotyledons, and primary and secondary needles all were susceptible at certain ages. Percentage infection of all inoculated organs decreased with increasing age. Stem galls developed following inoculation of all organs except secondary needles. Average longitudinal growth of the fungus in infected organs was relatively uniform for a given organ, but growth of the fungus in the hypocotyls and stems after 7 or 8 wk was more than double that in the foliar organs. Patterns of colonization of the different organs by *C. fusiforme* are described.

Additional key words: Fusiform rust, *Pinus elliottii* var. *elliottii*, histology.

Fusiform rust, which is caused by *Cronartium fusiforme* Hedgc. & Hunt ex Cumm., is an extraordinarily destructive disease of loblolly (*Pinus taeda*) and slash (*P. elliottii* var. *elliottii* Engelm.) pines in the southern United States. Although this disease is readily controlled in nurseries through proper application of appropriate fungicides, the only control that is economically feasible in the field is genetic resistance. The progeny of trees selected for putative resistance are screened by inoculating 4- to 6-wk-old seedlings with basidiospores of *C. fusiforme* and evaluating disease development after 9 to 12 mo.

A thorough understanding of infection phenomena and disease development in seedlings with little or no apparent resistance is needed to recognize and evaluate resistance. Necessary information includes the determination of potential sites of infection, changes in susceptibility of different infection sites with age, the rate and pattern of development of the pathogen in infected seedlings, and the time lapse between inoculation and the beginning of gall development.

The objectives of our research were to determine in susceptible slash pine: (i) relative importance of the hypocotyls, stems, cotyledons, primary needles, and secondary needles as sites of infection for the basidiospores of *C. fusiforme*; (ii) changes in susceptibility of the organs with maturation; (iii) relative rates of spread of the fungus in the different organs; and (iv) patterns of colonization in different organs.

MATERIALS AND METHODS

Pine culture and inoculation procedures.—Seeds of slash pine from a mixed seedlot were planted in greenhouse flats containing a mixture of equal volumes of sand and peat moss. When the hypocotyls were about 2.5 cm long, the seedlings were transplanted into 10-cm diameter pots containing a fumigated mixture of soil, sand, and peat moss (2:1:1, v/v) with two seedlings per pot.

Northern red oak (*Quercus rubra* L.) seedlings were inoculated by applying aeciospores diluted with talc to the lower surface of the leaves with a camel's-hair brush. The aeciospores were from a mass isolate collected in a slash pine plantation in Bleckley County, Georgia, in 1965 and stored according to the method of Roncadori and Matthews (12). The inoculated oak seedlings were

incubated in a mist chamber for 24 hr at 20 C and then moved to a greenhouse and maintained under natural light and sufficient artificial light for a 14-hr photoperiod per day. Oak leaves with telia of *C. fusiforme* were harvested for the collection of basidiospores between 4 and 6 wk after inoculation.

The relative importance of the different organs of slash pine seedlings as sites of infection for *C. fusiforme*, and the changes in susceptibility of these organs with age, were determined by inoculating the organs on seedlings of different ages with basidiospores of *C. fusiforme* (Table 1). The age of seedlings was measured from the time seeds were planted. All seedlings of a given age and point of inoculation were inoculated on the same day. At least 90 seedlings were inoculated for each combination of age and point of inoculation. Forty seedlings of each age-point of inoculation combination were inoculated for histological examination. A minimum of 50 additional inoculated seedlings of each age-point of inoculation combination were maintained in the greenhouse for observation of gall development.

The inoculation procedure has been described (8). Briefly, the basidiospores were collected and concentrated onto Millipore filter disks and transferred from the filters to the point of inoculation with an instrument constructed from glass capillary tubing. Inoculated seedlings were placed in a mist chamber at 20 C and incubated for 54 hr. The seedlings were then moved to a greenhouse and maintained under natural light and sufficient artificial light for 14 hr of continuous light per day.

Histological procedures.—The inoculated organs of the seedlings were collected for histological examination at 1-wk intervals for 8 wk beginning 1 wk after inoculation. Since five inoculated seedlings were collected each week, the numbers of organs examined per week were five hypocotyls or stem specimens, 30 to 45 cotyledons, 20 primary needles, and 40 secondary needles. The material collected for histological examination was fixed in formalin, propionic acid, and ethyl alcohol, dehydrated with tertiary butyl alcohol (5), and embedded in Tissuemat (melting point 56.5 C) (Fisher Scientific Company Pittsburgh, PA). Specimens were sectioned serially at 15 μm on a rotary microtome, stained with periodic acid-Schiff reagent (2), and mounted serially in Permount (Fisher Scientific Company Pittsburgh, PA).

Each sectioned organ was examined microscopically for infection, patterns of fungal growth, host response, and total longitudinal growth of the fungus. The latter was determined by measuring and summing the proximal and distal extension of hyphae from the point of infection (poi). In the cotyledons, growth of the fungus from the poi toward the stem only was also measured.

TABLE 1. Design of the inoculation experiment, influence of organ and age on infection, and average growth of mycelium in tissues of slash pine seedlings after inoculation of different organs with basidiospores of *Cronartium fusiforme*

Organs inoculated	Age of seedling at inoculation (days)[a]	Numbers of seedlings inoculated	Percentage of plants infected[b]	Average total longitudinal growth of fungus (mm)[c]
Hypocotyls, 1 cm below cotyledons	17	210	64	17.2
	29	130	19	16.6
	51	130	0	0.0
	135	90	0	0.0
Cotyledons, 1 cm from stem[d]	29	130	39	6.7
	51	130	4	7.6
Stems, 1 cm above cotyledons	51	130	43	13.6[e]
	135	90	0	0.0
Primary needles, 1 cm from stem[f]	51	130	5	8.0[e]
Stems, between 2nd and 3rd fascicles of secondary needles	135	90	35	17.9
Secondary needles, 1 cm from stem[g]	135	90	66	4.0[e]

[a]Days after planting seed.
[b]Infection determined by histological examination and gall development.
[c]Total growth measured both proximally and distally from the point of infection to the furthermost extension of hyphae after 8 wk, unless otherwise noted.
[d]All cotyledons inoculated.
[e]Seven weeks' growth.
[f]First four needles.
[g]First four fascicles of needles formed were inoculated per plant, two needles per fascicle.

RESULTS

Inoculation technique.—None of the inoculated organs showed major injury to the surface cells due to inoculation. Although a few cotyledons from plants inoculated at 29 days had injuries that may have resulted from the inoculation procedure, that kind of damage was infrequent and did not appear to alter penetration and infection.

Influence of age of seedlings and organs at the time of inoculation.—All organs were susceptible at some stage of maturation. However, the percentage of seedlings that became infected varied with the organ inoculated and the age of the seedlings at the time of inoculation (Table 1). The highest percentage of stem and hypocotyl infections resulted from inoculation of the hypocotyls when the seedlings were 17 days old. Similar inoculations of 29-day-old seedlings caused infection less than one-third as often. None of the seedlings inoculated on the hypoctoyls at 51 and 135 days became infected.

The percentage of 51-day-old seedlings that became infected after being inoculated on the stem 1 cm above the cotyledons was lower than after hypocotyl inoculations at 17 days, but more than double the hypocotyl rate at 29 days (Table 1). No infection occurred on the 135-day-old seedlings inoculated at the same stem position. However, inoculation of stems at 135 days in the area where fascicles of secondary needles were developing resulted in 35% infection.

Inoculation of the three foliar organs produced quite variable results. The susceptibility of cotyledons followed a pattern with age similar to the hypocotyls and stem inoculations. A difference of 22 days in maturity of the cotyledons (29-51 days) resulted in a decrease from 39% to 4% of seedlings infected (Table 1). The highest percentage infection (66%) occurred in secondary needles. Only 5% of the 130 inoculated seedlings had one or more infected primary needles.

Fungal growth rates.—Average longitudinal growth of *C. fusiforme* in hypocotyls and stems generally was similar, regardless of the age at inoculation or the point of inoculation (Table 1). In the foliar organs, however, the average growth of the fungus was considerably less than in hypocotyls and stems. Average growth in foliar organs was greatest in cotyledons and primary needles and least in secondary needles, in which it averaged only 4 mm in 7 wk. During the same 7 wk, the needles grew an average of 68 mm.

The average longitudinal growth of the fungus from the poi toward the stem was measured in infected cotyledons that were sectioned and examined histologically. After 8 wk, growth from the poi toward the stem averaged 3.6

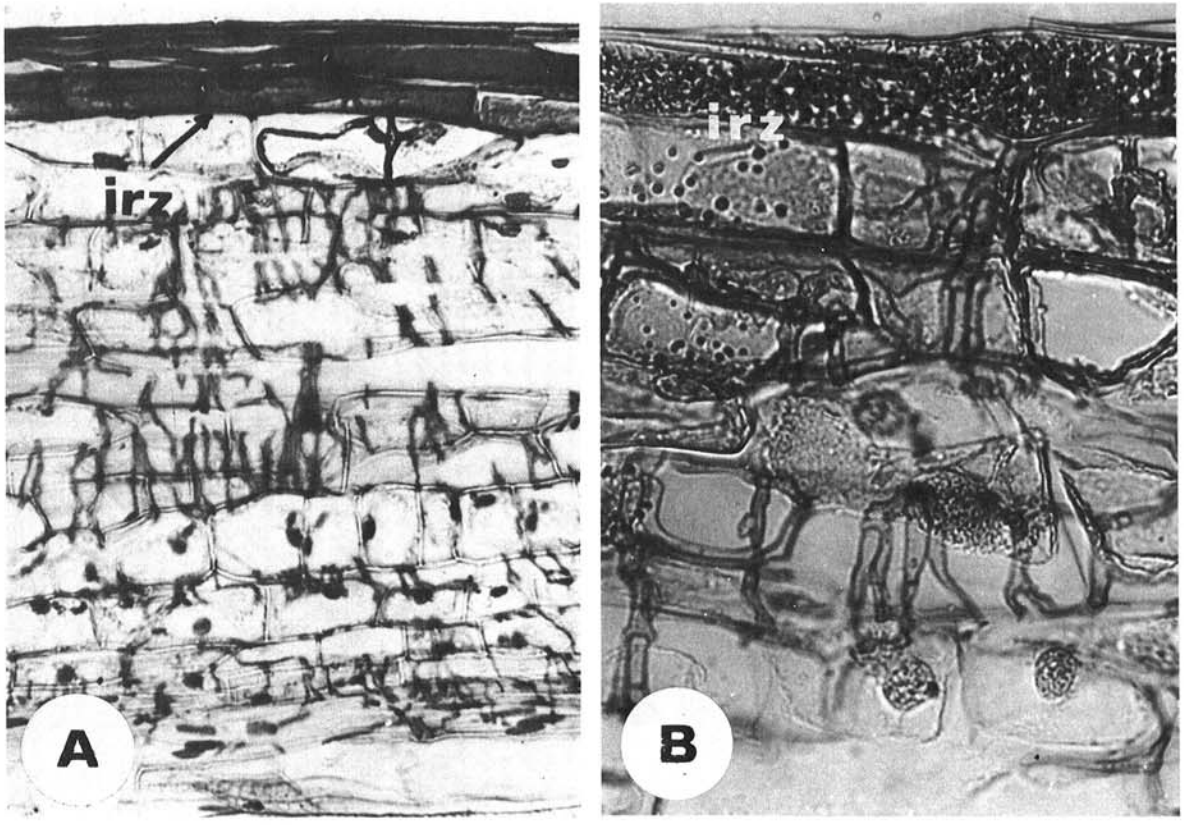

Fig. 1-(A. B). Early colonization patterns by *Cronartium fusiforme* in hypocotyls of slash pine seedlings (longitudinal sections). **A)** Development of the fungus in a hypocotyl from the point of infection and the initial response zone (irz) after 1 wk (×125). **B)** Fungus hyphae spreading as multiple strands from the point of infection and irz (×500).

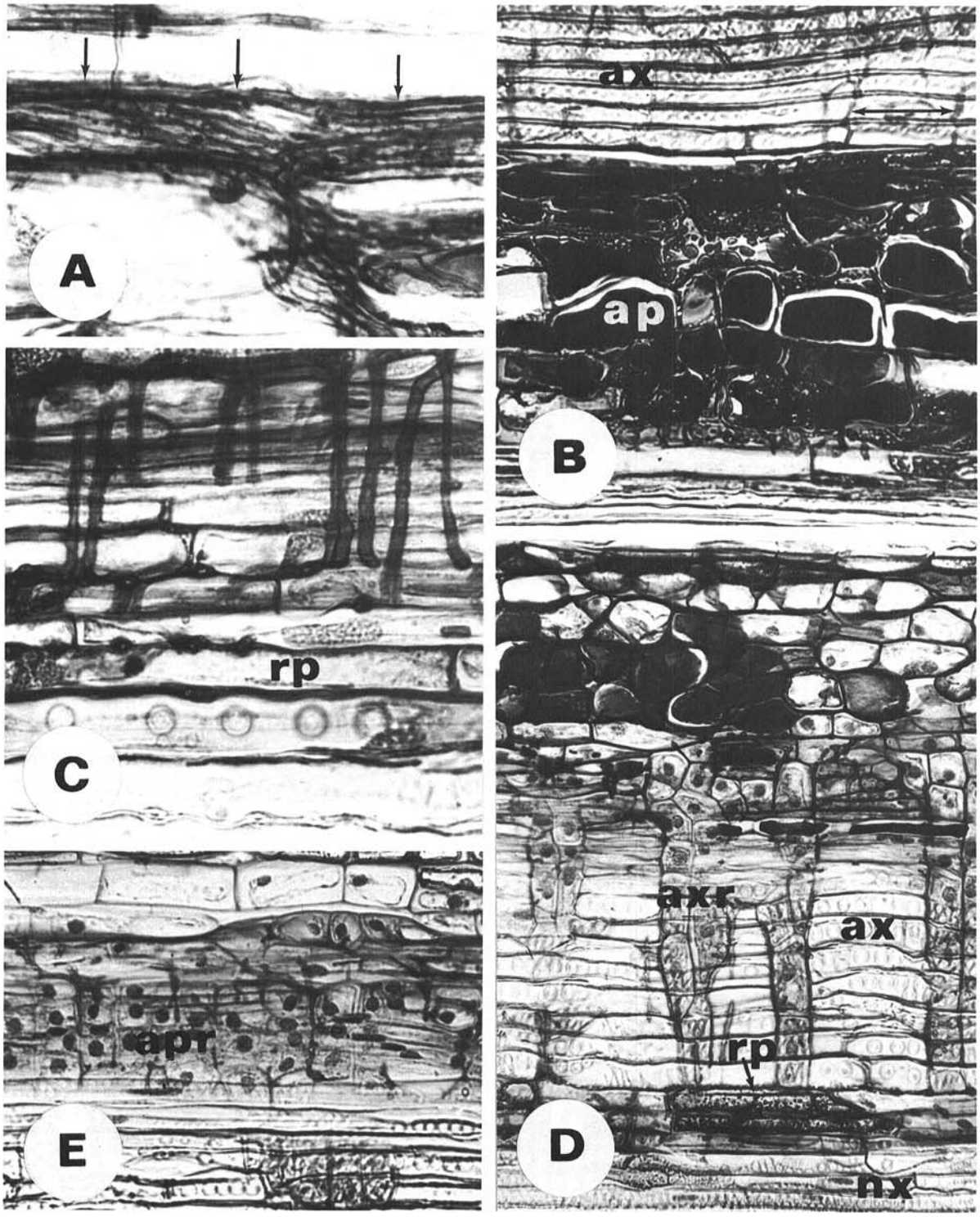

Fig. 2-(A to E). Growth of *Cronartium fusiforme* in hypocotyls of slash pine seedlings and host responses (longitudinal sections). **A)** Longitudinal spread of the fungus as multiple strands of hyphae (arrows) in the cells of the cortex (×500). **B)** Abnormal pith cells (ap) produced following colonization by the fungus and hyphae (arrow) extending from the pith across the abnormal xylem (ax) (×310). **C)** Reaction parenchyma (rp) (×500). **D)** Hypocotyl 5 wk after infection; note reaction parenchyma (rp), normal xylem (nx), abnormal xylem (ax), and abnormal xylem ray cells (axr) (×310). **E)** Abnormal phloem ray cells (apr) (×310).

mm and ranged from 1.2 to 5.2 mm.

Development of stem galls.—Ten months after inoculation, the percentages of seedlings on which typical galls were observed, ranked by point of inoculation, were: hypocotyls 52%, stems 34%, cotyledons 47%, primary needles 12%, and secondary needles 0%. Even after 15 mo, none of the seedlings with infected secondary needles developed galls.

Colonization of hypocotyls.—One week after inoculation of hypocotyls, basidiospores had germinated and produced short germ tubes that formed appressoria. Penetration was direct, primary hyphae were formed in the cells of the epidermis, and intercellular hyphae originating from a primary hypha in an epidermal cell spread rapidly into the cortex either as thin strands in a fanlike pattern or in multiple strands that developed perpendicular to the epidermis (Fig. 1-A, B). In most specimens the innermost growth of the hyphae extended to within a few cortical cells from the cambial area. In the area immediately adjacent to the poi, the cells were intensively stained and had a distinct granular appearance. The heavily stained areas will be referred to subsequently as initial response zones (irz).

Two weeks after inoculation the volume of hypocotyl tissue colonized by the fungus was more than double that invaded after 1 wk. The hyphae at and near the periphery of the colonized areas were thin and relatively sparse with only a few small haustoria. In and around the irz, however, the fungus had proliferated. The irz had increased in size, and large haustoria were in almost all cells. Haustoria and intercellular hyphae had increased from the margin of the irz to about the limits of colonization after 1 wk. The maximum radial penetration of hyphae observed after 2 wk was generally to the cambial area.

During the 3rd and 4th wk after inoculation, longitudinal spread of the fungus was mainly intercellular in the inner cells of the cortex and along the phloem and cambium, often as multiple strands of hyphae (Fig. 2-A). The radial growth of the fungus during this period was to the cambial area in most specimens. In a few specimens, however, the fungus had grown into the pith. Hypertrophy of affected cortical cells was observed in and around the irz 3 wk after inoculation.

By the end of the 4th wk after inoculation, haustoria were apparent in most cells in all but the most recently colonized areas. The irz in most specimens reached final size 4 wk after inoculation. The cambium was completely formed in all specimens and was producing normal secondary xylem that had not been affected by the presence of the actively growing fungus.

After 5 wk, the fungus was well established in the cambial region and had stimulated the production of modified secondary xylem in some specimens. The first indications of hypertrophy and hyperplasia were evident mainly in the inner cells of the cortex and phloem. The fungus was well established in the cambium of all specimens and also was present in the pith of some (Fig. 2-B). Hypertrophy was evident in the infected pith, and all cells of the secondary xylem between the pith and the poi were distorted. Strands of hyphae extended from the cambial region, across the secondary xylem, and into the pith.

In the seedlings where the fungus had not grown into the pith, maximum radial progress of the fungus into the tissues was marked by the development of one to three strands of cells referred to by Jewell et al. (4) as reaction parenchyma (Fig. 2-C, D). Secondary xylem cells that were formed prior to the time the fungus reached the cambium and stimulated the production of reaction parencyma were not distorted, but those formed later were definitely abnormal. These cells were irregular in shape and generally much wider and shorter than cells of noninfected xylem (Fig. (Fig. 2-D). There was increased hypertrophy of affected cortical cells and both hypertrophy and hyperplasia of the phloem. Phloem ray cells were larger and more numerous at and around the area where the fungus had first reached the cambium. In some specimens, an almost continuous layer of phloem ray cells extended longitudinally in both directions from a point radially perpendicular to the poi to the limits of reaction parenchyma development (Fig. 2-E). Abnormal xylem ray cells developed in the same general area (Fig. 2-D).

Initial gall development occurred only on the side of the hypocotyl that was inoculated. In most specimens, the fungus also penetrated tangentially to the opposite side of the stem but caused no distortion or proliferation of cells. Longitudinal growth of the fungus was confined to the cambial region, phloem, and the innermost two or three cells of the cortex. After gall initiation, the patterns of growth of the fungus and the production of abnormal host tissues were as described by Jewell et al. (4) on 1-yr-old seedlings.

The general pattern of infection and colonization in hypocotyls inoculated at 29 days was similar to that after inoculation at 17 days. An irz formed at the poi, and the fungus grew radially from this zone into the cambial region. Longitudinal spread was mainly in the phloem and inner cortex. Two differences observed between the seedlings of the two ages were the quantity of mycelium in the tissues and the amount of gall development after 8 wk. Hypocotyls inoculated at 29 days usually contained less mycelium and fewer haustoria, especially in the regions radial and longitudinal to the poi, than those inoculated at 17 days. The hyphae increased under and around the poi after 4 wk, but this buildup of the fungus did not involved as large an area as in the specimens inoculated at 17 days.

After 8 wk, host responses were less conspicuous in the hypocotyls inoculated at 29 days than in those inoculated at 17 days. The fungus reached the cambium during the 3rd and 4th wk after inoculation, and reaction parenchyma were evident after 4 wk. The tracheids laid down outside the reaction parenchyma were distorted. Phloem cells produced in the infected regions were as described for hypocotyls of seedlings inoculated when 17 days old. Very little distortion and proliferation of cortical cells and few ray cells were evident after 8 wk. In general, gall development 8 wk after inoculation of 29-day-old seedlings was less advanced than after 5 wk in seedlings inoculated at 17 days. After 14 months, however, the galls on seedlings inoculated at 29 days were the same average length and diameter as galls on seedlings inoculated at 17 days.

Colonization of stems.—In the stems, irz formed 1 wk after inoculation, but were smaller, stained less intensely, and did not have the granular appearance of the irz in the

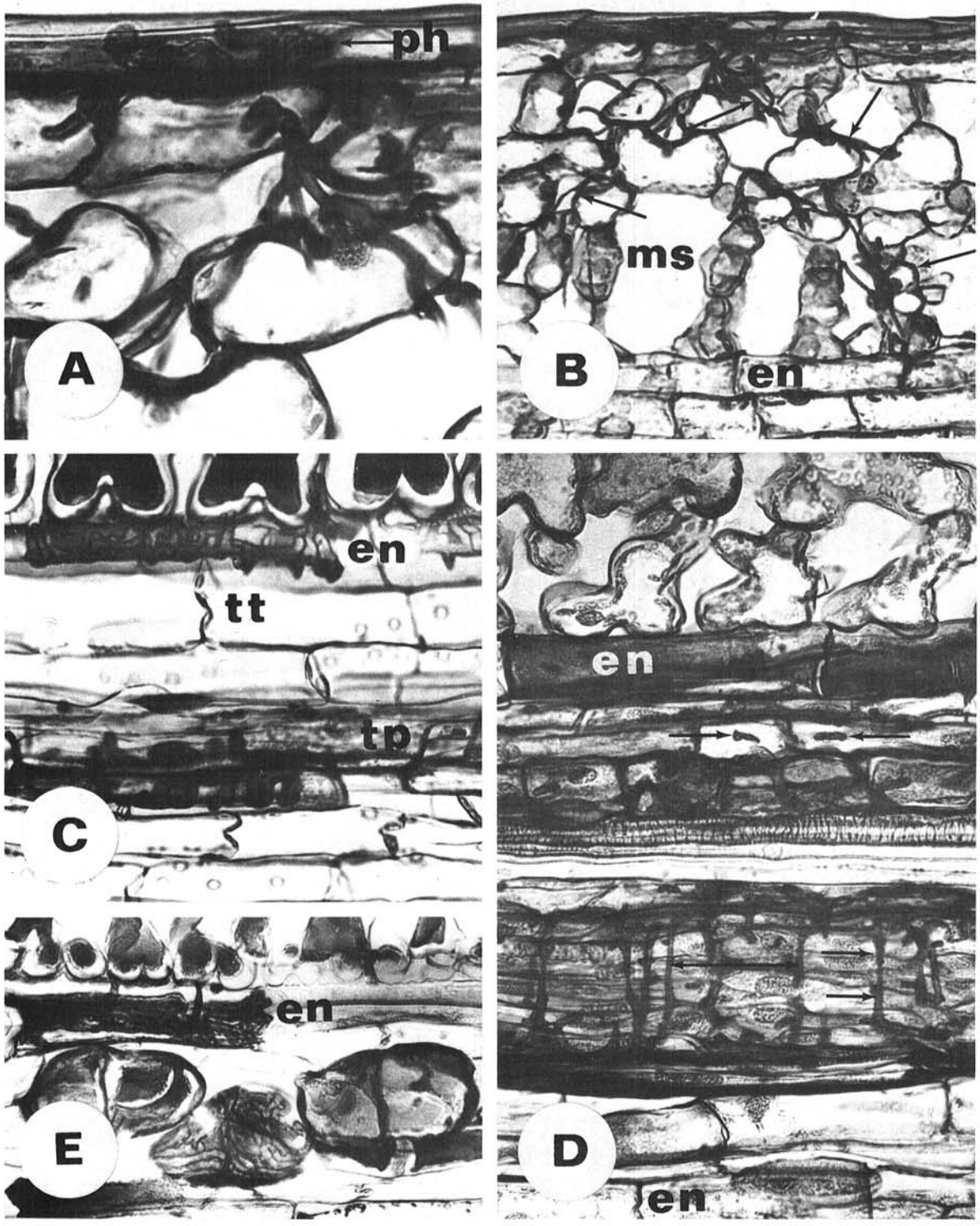

Fig. 3-(A to E). Typical development of *Cronartium fusiforme* in cotyledons after 1 wk(longitudinal sections). **A)** Growth of secondary hyphae from the primary hyphae (ph) in an epidermal cell (×310). **B)** Total development of the fungus after 1 wk with growth of mycelium (arrows) through the mesophyll (ms) to the endodermis (en) (×125). **C)** A cotyledon 2 wk after inoculation; note buildup of fungus at the endodermis (en) and the transfusion parenchyma (tp), and absence of the fungus in the transfusion tracheids (tt) (×310). **D)** Colonization of the vascular tissues by the fungus; note hyphae and haustoria (arrows) in most tissues enclosed by the endodermis (en) (×310). **E)** Hyphal masses in the endodermis (en) and in enlarged cells of the transfusion parenchyma (×310).

hypocotyls. Well-formed and distinctive irz similar to those in the hypocotyls did not develop in the infected stems until 6 wk after inoculation.

The other difference between hypocotyls and stems was in the time between inoculation and initiation of gall development. Gall development was evident in the infected stems 3 wk after inoculation, 2 wk sooner than in the hypocotyls. Reaction parenchyma formed, and three to five cells of distorted xylem were present between the reaction parenchyma and the cambium. Once galls were initiated, the patterns of development were the same in both hypocotyls and stems.

Colonization of cotyledons.—Cotyledons were infected by direct penetration and formation of primary hyphae in epidermal cells (Fig. 3-A). Secondary hyphae then developed and initiated colonization. At the end of 1 wk, hyphae had ramified through the cells of the mesophyll to the endodermis (Fig. 3-B).

After 2 wk, the mesophyll cells in the infected areas were generally rounded in comparison to normal, rectangular, convoluted mesophyll cells. Hyphae had spread radially past the endodermis and into strands of parenchyma two to three cells inside the endodermis (Fig. 3-C). Longitudinal spread of the fungus occurred at the endodermis and in the vascular parenchyma. Hyphae also spread for some distance around the periphery of the vascular cylinder in the area of the endodermis and innermost cells of the mesophyll.

After 3 wk, the fungus was growing mainly in the area of the endodermis and inner mesophyll; it had not advanced radially beyond the first strands of parenchyma inside the endodermis. The amount of mycelium increased somewhat around the initially invaded endodermal cells and in the first parenchyma cells colonized beyond the endodermis.

After 4 wk, the fungus had encircled the endodermis and was well established inside the vascular cylinder in a number of different tissues (Fig. 3-D). Haustoria were observed in the transfusion parenchyma and the phloem, and individual strands of hyphae had grown radially against the sides of cells of the protoxylem and were flattened against the cell walls. Hyphal strands had grown radially and longitudinally between xylem cells, and small haustoria were present in some xylem cells. Longitudinal growth of *C. fusiforme* was confined to cells enclosed by the endodermis. Mycelium was intercellular along the cells of the parenchyma and phloem. Mesophyll cells at and adjacent to the poi were intensely stained, distorted, and many were filled with granular substances. The hyphae and haustoria in the centers of these regions were generally encrusted and appeared to be degenerated. At the margins of these areas, the hyphae and haustoria appeared normal. No proximal or distal growth of the fungus was observed in the mesophyll after 4 wk.

In many of the infected foliar organs after 4 wk, some cells of the infected vascular parenchyma had undergone hypertrophy and become generally elliptical in shape (Fig. 3-E). Many of these distorted cells contained masses of hyphae that may be similar to those described previously in needles of white pine infected by *Cronartium ribicola* (1, 10).

After 5 and 6 wk, the pattern of colonization was similar to that described after 4 wk. The only differences observed were more mycelium at the endodermis just beneath the point of infection and greater longitudinal spread along the vascular tissues.

After 7 wk, there were three distinct zones of colonization. In the first, which encircled the endodermis, mesophyll cells at and around the poi were heavily colonized with hyphae and numerous haustoria. This zone was widest near the endodermis. The mesophyll cells were distorted, darkly stained, granular, and appeared generally moribund, especially in the center of the zone. Hyphae and haustoria in the center of this area were encrusted and collapsed. The second zone started at the interface of mesophyll and the endodermis. The fungus was observed in the endodermis and in almost all tissues enclosed by the endodermis. The third zone was at the extreme limits of longitudinal spread. Here the fungus occurred as hyphae. Haustoria were not observed in these most recently invaded areas.

After 8 wk, colonization was similar to that after 7 wk. An increase in the amount of mycelium was observed in and around the parenchyma cells and vascular regions in zone 2. The hypertrophy of the parenchyma cells had increased, and some of the transfusion tracheids were crushed in the regions of maxium distortion. The fungus had not spread longitudinally in the mesophyll between 7 and 8 wk. Most of the mesophyll cells in the infected areas apparently were dead, had collapsed, and were disintegrating.

Between 4 and 8 wk after infection, the longitudinal spread of the fungus was confined to tissues encircled by the endodermis. This was the case until the fungus reached the stem. Once it had penetrated the stem, the fungus spread rapidly from the cotyledon or primary needle trace into the adjacent tissues of the stem or hypocotyl.

Colonization of secondary needles.—Penetration by *C. fusiforme* into the secondary needles was direct, but occurred almost exclusively through some portion of the stomatal apparatus. Basidiospores that had lodged in stomates germinated and penetrated either the guard or subsidiary cells. Apart from this association with stomates, development and colonization by the fungus were generally the same as in the cotyledons. The same pattern of hypertrophy occurred in the vascular tissues, but the increase in size of cells was not as great as that observed in the infected cotyledons. The fungus did not grow as rapidly in the vascular system of the secondary needles as in the cotyledons and did not reach the stem in any of the inoculated seedlings.

DISCUSSION

Hypocotyls, cotyledons, stems, and both primary and secondary needles all were suitable for infection by *C. fusiforme* if inoculated before a certain stage of maturation. Percentage of infection decreased markedly with increasing maturity of all organs that were inoculated at different ages (Table 1). Since basidiospores generally germinated on all organs regardless of age, the decrease in percent infection apparently resulted mainly from failure of germ tubes to penetrate. Infections that occurred on more mature organs developed similarly to those in the younger material, with some differences in intensity of initial colonization. The low percentage of

infections on primary needles undoubtly reflected the maturity of the needles at the time of inoculation. Numerous subsequent inoculations, not reported here, with the same and other methods, resulted in much higher rates of infection of primary needles less mature than those inoculated in this study.

The infection of secondary needles is of interest for two reasons. First, this is believed to be the first report of infection of these organs by *C. fusiforme*; second, the infections occurred through some portion of the stomatal apparatus rather than directly into other cells of the epidermis as occurred on all other organs. The presence of the basidiospores in the stomatal cavities may have been due exclusively to the inoculation technique, by which spores were forced into the stomates as well as being deposited on the needle surfaces. It is possible that basidiospores are not deposited in the stomates in nature, although the stomatal openings in slash pine are large enough to permit their passage. If infection occurs near the fascicle sheath before the needles have completed elongation, the fungus may be able to grow into the stem and produce a gall. Secondary needles must be considered as a potential site of infection in nature.

A decrease in susceptibility of the different organs of slash pine to infection occurs as they mature. During growth periods, a zone of potentially susceptible tissues extends from the apical meristem downward to where maturation inhibits infection. As the seedling grows, this zone moves upward. This constantly changing zone of susceptible tissue is an important variable that must be considered in determining the frequency of application of protective fungicides in southern pine nurseries (6) and in selecting the appropriate age for inoculating seedlings being screened for resistance to *C. fusiforme*.

For given organs, the extent of colonization by *C. fusiforme* 7 or 8 wk after inoculation did not vary greatly among seedlings. Apparently, if the fungus infects an organ and is not restricted by a host response at the site of infection (9), it grows through the tissues at about the same rate irrespective of age of the organ at the time of inoculation.

Growth was more rapid in hypocotyls and stems than in the foliar organs. The slower growth of the fungus in the cotyledons and primary needles is of interest in evaluating the relative importances of these organs as sites of infection on slash pine seedlings. With a maximum growth rate of about 0.7 mm/wk, infections in primary needles and cotyledons more than 1 cm from the stem probably seldom reach the stem before the infected foliar organ dies. After injecting basidiospores into foliar organs of slash pines, Powers (11) came to the same conclusion.

Although the rate of spread of the pathogen and the pattern of colonization were quite similar in the organs of different ages, there was a distinct difference in the initiation of galls. The first evidence of gall development in older hypocotyls and stems occurred 1 to 3 wk before such response in younger hypoctyls. A likely explanation for this difference is that the vascular cambium was not complete in the younger hypocotyls when the pathogen reached that area. Therefore, gall tissues were not initiated until the cambium was completely formed and functioning.

The rates of growth of the fungus, the sequence of tissues colonized, and the host responses to infection in the three different foliar organs were examined for responses similar to the resistant reactions that have been reported in needles of white pines infected by *C. ribicola* (3, 7). If similar reactions occur in the infected foliar organs of slash pine seedlings infected by *C. fusiforme*, there was no evidence of it in the population of seedlings examined in this study.

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