Types of Germination and Differentiation of Vesicles by Basidiospores of Cronartium ribicola

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Research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, by the National Science Foundation through appointment of the senior author as a NSF Fellow for 1971 and 1972, and by McIntire-Stennis Project No. 1858. Appreciation is expressed to Steven A. Vicen for assistance in preparation of the illustrations.

Accepted for publication 16 April 1975.

ABSTRACT

Germination of basidiospores of *Cronartium ribicola* on artificial substrates and eastern white pine needle surfaces resulted in various types of germ tube growth and development. In what was considered normal germination, one to several thin germ tubes, 1-3 μm in diameter, grew from each spore. Only one continued to elongate, usually reaching a length of 100-300 μm, and occasionally forming one or two short branches. Germ tubes usually grew along the surface of the substrate, but in moisture chambers often grew into the air from both needles and artificial substrates. Elongation was greatest between 9 and 15 C, but dropped sharply at higher temperatures. Intercalary or terminal swellings (vesicles) often were developed, which closely resembled the infection vesicle that was produced in the substomatal chamber during successful needle penetrations. On collodion membranes at 16 C, less than 5% of germ tubes differentiated vesicles. Fluctuating temperatures, high temperature (28 C) shocks of 1 or 2 hours duration, and changes in osmotic concentration of a supporting solution, significantly increased the proportion of vesicles formed. The duration of incubation at 16 C prior to temperature shock affected both the number and type (terminal or intercalary) of vesicles. Vesicle formation was hypothesized to be a result of disrupted growth following a change in the germ tube environment. Basidiospores associated with water droplets usually formed thick (3- to 5-μm) germ tubes or sterigmata and secondary basidiospores. Sometimes a zigzag type of germ tube growth occurred, characterized by an angular pattern of the main germ tube axis and the presence of numerous short side branches. Such development was induced by a volatile substance associated with germinating spores and was modified by the nature of the substrate and an unknown factor associated with the time of year. Zigzag growth prevented vesicle formation, reduced effective germ tube length, and inhibited nuclear migration and division.

Phytopathology 65:1061-1071

Additional key words: spore germination, white pine blister rust, zigzag growth, secondary spores.

Cronartium ribicola J. C. Fischer ex Rabenh., the cause of white pine blister rust, infects five-needled pines through stomata, and in the process forms a substomatal vesicle (37). With programs to develop rust-resistant planting stock well under way in both the eastern and western United States, it is important to understand as clearly as possible the factors which influence infection and mechanisms of disease resistance. Several mechanisms for resistance to white pine blister rust have been identified which involve needle reactions (20, 22, 23, 27, 32, 33, 35). Continued work on the nature and expression of resistance demands reliable inoculation techniques and better knowledge of the infection biology of the pathogen, including factors influencing basidiospore germination and differentiation of infection structures.

Early reports on *C. ribicola* basidiospore germination emphasized the abundant formation of secondary spores and short, thick, germ tubes (5, 6, 48). These workers, as well as Hirt (21) and Bega (2), germinated spores on agar or moist glass surfaces. Hirt (21) asserted that at temperatures from 10 to 20 C basidiospores normally germinated to form secondary spores which in turn formed thin, elongating germ tubes. Bega (2) reported that pH as well as temperature influenced the amount of secondary spore formation on water agar surfaces. Observations of *C. ribicola* basidiospore germination on host needle surfaces revealed predominantly thin, elongate germ tubes, although secondary spores and thick, short germ tubes were also reported (2, 35, 38).

A pattern of zigzag germ tube growth, characterized by numerous short branches along an angular or zigzag germ tube axis, has been described for germination of urediospores of several *Puccinia* spp. (10), but this has not been reported for *C. ribicola*.

Once it was determined that substomatal vesicles were associated with needle infection, the question of their significance was raised, and we felt that better knowledge of their formation would lead to a clearer understanding of the conditions which influence pine infection. Factors controlling formation of infection structures (appressorium, penetration peg, vesicle, and infection hypha) have been studied with germinated urediospores of several rust species. These include: thigmotropic stimuli (9, 11, 30), carbon dioxide concentration (53), nutritional factors (7, 17) volatile chemicals released from the spores (1, 51), and changes in light and temperature (16, 30, 46). So far, no theory of infection structure differentiation that encompasses all these diverse observations has been proposed. The first report on the differentiation of vesicles from basidiospores of *C. ribicola* germinated on artificial substrates, such as collodion and cellophane membranes, was given by Patton (36) in a discussion of previously unpublished work by the author and his co-worker Pritam Singh. The present work expands on these observations.

The purpose of this study was to examine the factors which influence basidiospore germination, germ tube development, and differentiation of infection structures on artificial substrates and host needle surfaces. This

information was utilized in subsequent work, to be reported later, for obtaining maximum infection levels by artificial inoculation of eastern white pine (*Pinus strobus* L.) seedlings as part of a continuing study of the infection biology and mechanisms of host resistance to *C. ribicola*.

MATERIALS AND METHODS.—*Cronartium ribicola* was maintained in the uredial state on *Ribes nigrum* L. Urediospores from several areas in Wisconsin were mixed for the original inoculation. Ribes were spray-inoculated with water suspensions of urediospores and incubated in a dew chamber at 16 C for 24 hours. After incubation, Ribes plants were held at 24 C for 10 to 14 days for uredial development, and then at 16 C for an additional 14 days for telial formation. Teliospores remained viable on attached Ribes leaves for at least 2 months.

Collodion membranes (30) were usually used as the germination substrate for basidiospores. Five milliliters of a solution of collodion, ethyl ether, and 100% ethanol (1:3:1, v/v) were poured into a 14-cm diameter petri plate and swirled. The plate was then set upright to drain and dry for 15 minutes. The residual film was cut into 2 cm squares which were floated from the glass on distilled water.

Spores were deposited on membranes in settling-tower moist chambers. Milk cartons [1.9-liter (0.5 gallon)], with both ends removed, were lined with cheesecloth and set into water-filled 14-cm diameter petri plate halves. Telia-bearing Ribes leaves were soaked for 1 minute in cold water, shaken, then placed on 6.35-mm (0.25-inch) mesh screens atop the towers. The towers were covered with plastic bags and placed in a 16 C chamber. Spore cast began in about 12 hours. Collodion membranes floating on water in 9-cm diameter petri plate halves were placed beneath the towers for 1 hour spore-deposit periods, then covered and transferred to the appropriate incubation chamber.

Spores on floating membranes were incubated for 72 hours under various conditions. A Percival dew chamber with manual temperature control was used for most experiments. Temperatures were monitored in all indoor chambers with copper-constantan thermocouples and a Westronics portable recorder, and in an outdoor mist chamber with a hygrothermograph.

After incubation, membranes were transferred to glass slides for observations of spore germination. The membranes were dried, mounted in Patterson's mounting medium (12) with aniline blue and trypan blue, and germinated spores observed with bright-field and phase-contrast microscopy. For examination of nuclei, membranes were thoroughly dried on gelatin-adhesive coated slides (24). The germinated spores were fixed in Carnoy's solution, hydrolyzed in 5 N hydrochloric acid, and stained with Giemsa.

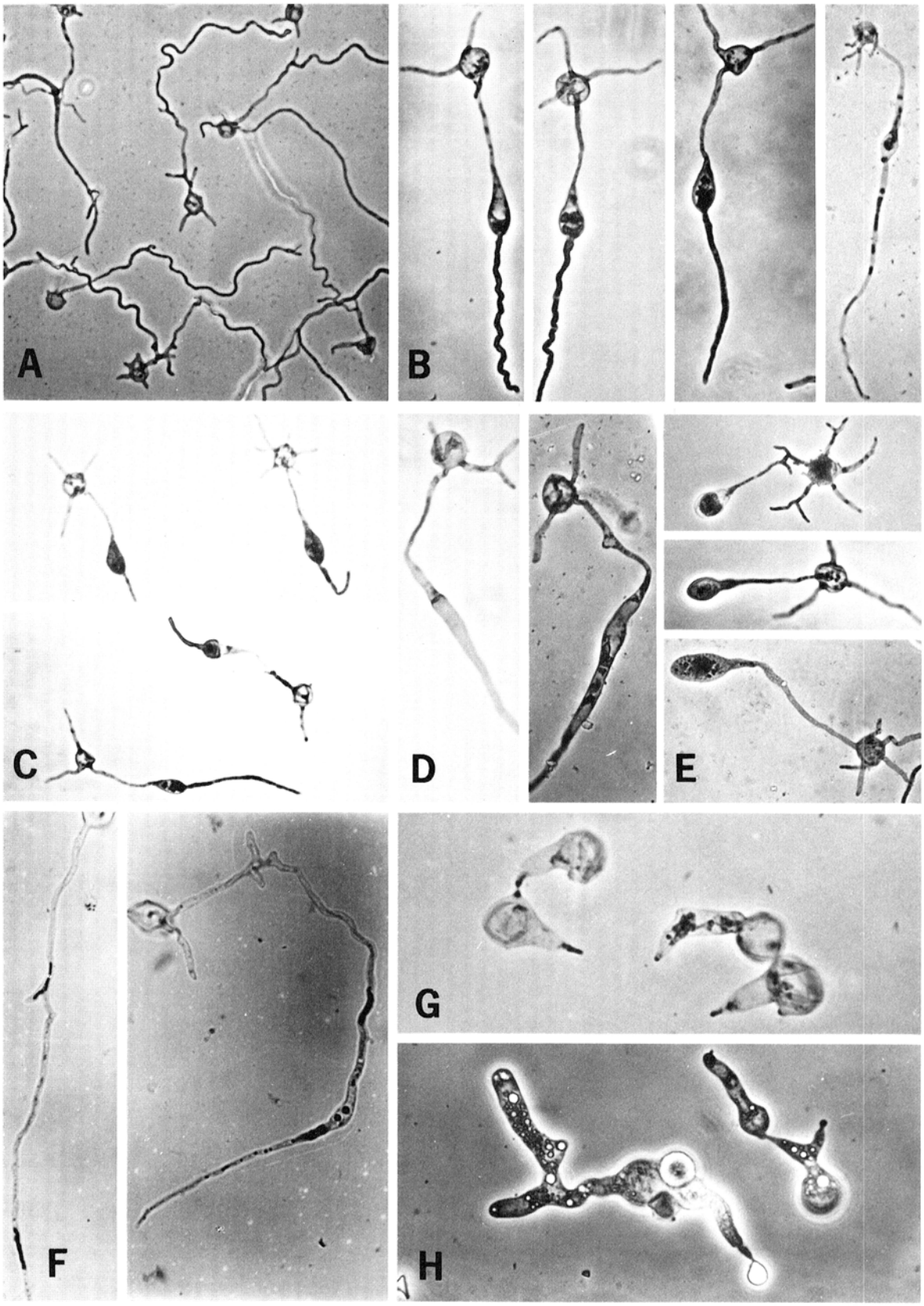
Spore germination on collodion membranes was compared to that occurring on needles of eastern white pine seedlings and transplants. An airstream inoculation apparatus similar to that developed by Snow (47) was used. In this system basidiospores abjected from telia on Ribes leaves were caught up in an airstream which passed through a narrow tube and impacted the spores in a discrete area on needles of the tree being inoculated. After inoculation, trees were covered with a moist plastic bag and incubated for 72 hours in a 16 C chamber. Observations of germinated basidiospores on needle surfaces were made with epifluorescence microscopy. Needles bearing germinated spores were dipped in an aqueous solution of optical brightener plus a drop of Tergitol Nonionic NPX (Union Carbide Corporation, New York). The brightener used was either (i) a 60 μg/ml solution of 4,4′-bis-4-aniline-6-bis(2-hydroxyethyl)-amino-s-triazin-2 ylamino-2-2′-stilbene disulfonic acid prepared from a 12% solution of the disodium salt in 42% aqueous Cellosolve, or (ii) a 250 μg/ml solution of Calcofluor White M2R New prepared from a 5% solution in 42% aqueous Cellosolve (both from American Cyanamid Co., Bound Brook, New Jersey). Brightened needles were mounted in water and observed with a Leitz Ortholux microscope equipped with a Ploem Vertical Illuminator (Leitz Wetzlar) with interchangeable dichroic mirrors.

Data on type of germination were based on counts from at least nine fields of view, for a total of at least 200 spores per membrane or needle. Spores on at least four replicate membranes or needles were counted for each test, with tests made two or more times. Average germ tube lengths were based on measurement of at least 50 germinated spores. Data were analyzed where applicable with Student's t test.

RESULTS.—The germination level of basidiospores exceeded 95% on collodion membranes and white pine needles in all tests where moisture and temperature conditions were favorable. Basidiospores germinated to form either (i) normal, thin relatively straight and unbranched germ tubes which were capable of forming vesicles, (ii) thick germ tubes or secondary spores, or (iii) zigzag germ tubes.

Normal germination.—On membranes and on host needles, more than 90% of the spores not associated with conspicuous water droplets formed one to several thin germ tubes, 1-3 μm in diameter (Fig. 1-A). Only one continued to elongate. Dominant germ tubes usually reached lengths of 100-300 μm, while the others grew 10-20 μm. Normal germ tubes grew in a straight or smoothly curving path, or occasionally in a wavy or corkscrew pattern. Germ tubes usually grew along the membrane or needle surface, but often germ tubes up to 300 μm long

Fig. 1-(A to H). Germination of basidiospores of *Cronartium ribicola* on collodion membranes after a 72-hour incubation period. **A)** Typical formation of thin germ tubes, one from each spore having become dominant. **B, C)** Several representative examples of typical pyriform vesicles that are similar to substomatal infection vesicles in white pine needles. **D)** Two examples of elongate vesicles. **E)** Three examples of typical terminal vesicles. **F)** Left, binucleate undifferentiated germ tube. Note basal nucleus extending into germ tube branch. Right, binucleate differentiated germ tube with a nucleus on either side of the vesicle. **G)** Left, basidiospore (top) germinated to produce a secondary basidiospore, which in turn has germinated to form a sterigma preparatory to the formation of another secondary basidiospore. Right, a basidiospore (top) germinated with a thick germ tube that terminated in a sterigma, and another basidiospore (bottom) formed a typical sterigma for production of a secondary basidiospore. **H)** Thick germ tubes with abortive sterigmata and elongate sterigmata forming secondary basidiospores.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grew away from both substrates under these incubation conditions.

Effect of temperature on germination and germ tube growth.—Basidiospore germination and germ tube elongation exhibited a sharp temperature response. After 1 hour of spore deposition at 16 C, spores on floating membranes were transferred to incubators at temperatures from 4 C to 36 C. The results of a representative trial are presented in Table 1. Germ tube elongation was greatest between 9 and 15 C.

TABLE 1. Effect of temperature on *Cronartium ribicola* basidiospore germination and germ tube growth on floating collodion membranes

Temperature-(C)	Terminal vesicles (%)	Germination (%)	Germ tube length (μm)
5	0	100	100
12	0	100	120
15	0	100	120
19	11	94	25
24	18	100	30
26	16	50	20
32	0	3	10
36	0	2	10

Germ tube growth rate at 16 C was determined by casting spores for 1 hour onto floating membranes and then removing groups of membranes at periodic intervals for measurement of germ tube length. Germ tubes formed within 2 hours of deposition, and growth continued for at least 72 hours. For the first 30 hours germ tubes elongated at an average rate of 3.5 μm/hour, and at a declining rate after that time.

Vesicle formation.—Vesicles that formed on collodion membranes and needle surfaces were either intercalary or terminal. Intercalary vesicles (with infection hyphae)

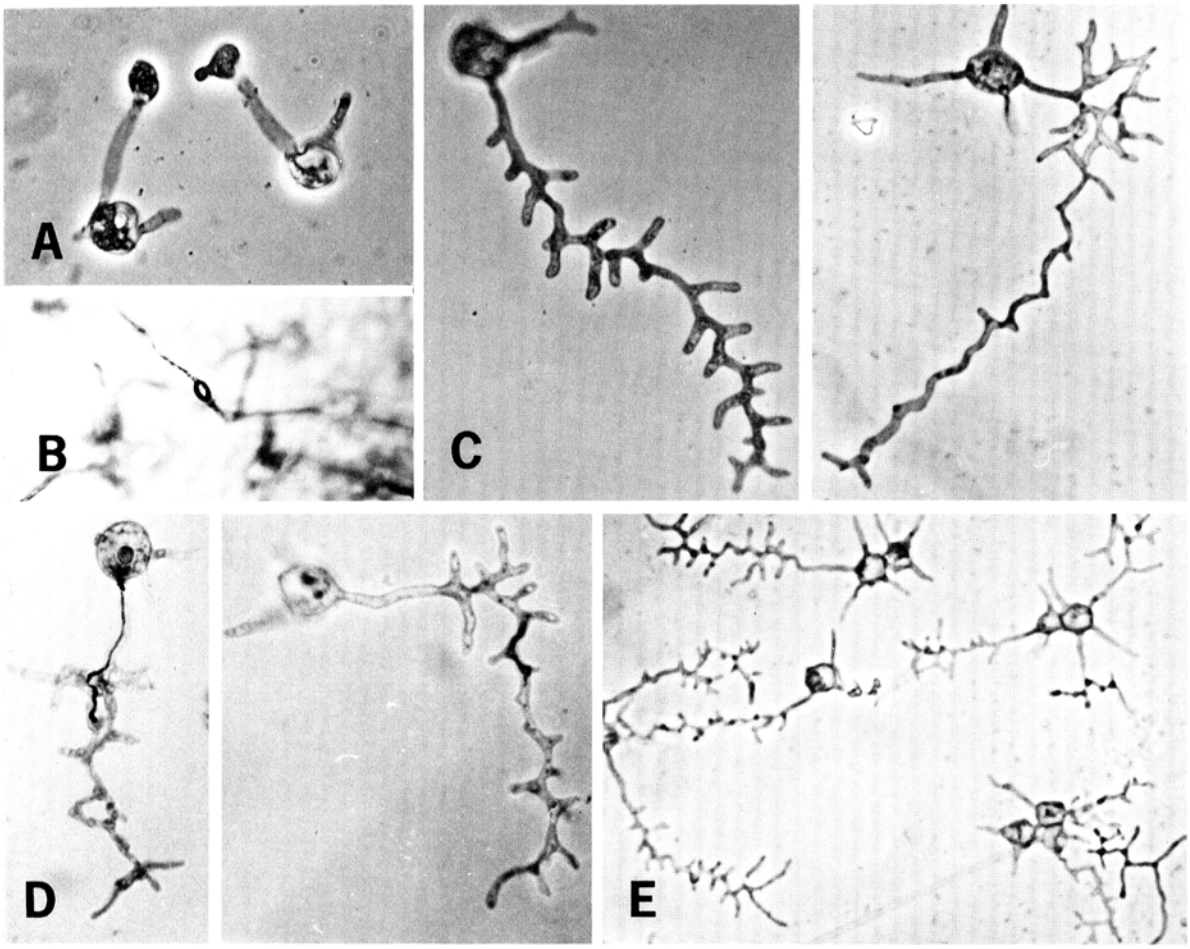

Fig. 2-(A to E). Germination of basidiospores of *Cronartium ribicola* on collodion membranes after a 72-hour incubation period. **A)** Irregular, terminal vesicles formed at fluctuating temperatures above 16 C. **B)** Typical intercalary vesicle formed on a germ tube that grew into the air above the substrate. **C)** Typical examples of zigzag germ tube growth. Left, continued growth from a single dominant germ tube. Note the angular path of main germ tube axis and the short side branches formed when new growing points arose behind the previous growing tip. Right, loosely-branched pattern as a result of irregular, zigzag growth from more than one growing point. **D)** Nuclei in zigzag germ tubes. Left, elongate nucleus extending into germ tube from the spore; right, a single relatively compact nucleus in the germ tube, bulging into germ tube branches. **E)** Typical zigzag development of germ tubes from spores at a relatively high density of approximately 300 spores per mm^2.

were similar to, but just as variable in shape and size as the vesicles produced during successful needle penetrations. Well-formed vesicles were pyriform, with the blunt end away from the spore, 8-10 μm in diameter and about 15 μm long (Fig. 1-B, C). An apparent crosswall often developed proximal to the vesicle. The single infection hypha which grew from intercalary vesicles was similar in size and shape to the germ tube before vesicle formation, except that branching was very infrequent. Many vesicles were irregular in shape, or elongate and narrower than the typical pyriform vesicles (Fig. 1-D).

Terminal vesicles were usually similar in size and shape to intercalary vesicles, but lacked infection hyphae (Fig. 1-E). Germ tubes that formed at temperatures above 16 C frequently developed terminal vesicles, which were smaller and more angular than those produced under more favorable conditions (Fig. 2-A).

Vesicle formation was induced on regular and paraffin oil collodion membranes (30), waterproof and nonwaterproof cellophane, 2% water agar, and the surface of *Pinus strobus* needles. Although vesicles usually differentiated on germ tubes growing along the substrate surface, they also often differentiated on long germ tubes growing directly away from the surface of membrane or agar substrates (Fig. 2-B).

Environmental influences on vesicle formation.—Since vesicles were differentiated by less than 5% of spores germinated on collodion membranes at a constant 16 C, several treatments were applied to test the effects of temperature or osmotic pressure on vesicle formation.

The effect of fluctuating temperatures was tested in an outdoor mist chamber, and in a programmed growth chamber. Outdoor mist chamber temperatures ranged from 16 to 18 C at night up to 27 C during the day. The growth chamber temperature fluctuated between 7 C and 22 C on a 12-hour cycle. Preliminary experiments at 16 C indicated that light had no effect on vesicle differentiation, and all subsequent growth chamber experiments were done without lights. Vesicle formation in the fluctuating temperatures of both the outdoor and controlled environment chambers was significantly increased (99.5% confidence limits) above the level observed on membranes held at 16 C (Table 2). Although there was no consistent difference in the total number of vesicles formed between growth chamber and outdoor experiments, the proportion of terminal vesicles was different. In the outdoor chamber 81% of the vesicles were terminal, but in the cooler growth chamber 45% of the vesicles were terminal, and on membranes held at a constant 16 C, 27% of the vesicles were terminal.

Temperature-shock experiments were designed to determine more precisely the role of high temperatures in vesicle formation. Spores were cast and incubated for various periods at 16 C, transferred to incubators at 24 or 28 C for 1 or 2 hours, and then returned to 16 C for the remainder of the 72-hour incubation period. Table 2 summarizes the results from 14 trials with temperature shocks given after an initial 16-hour incubation. The difference in total vesicle formation between shocked and nonshocked germinating spores was highly significant (99.5% confidence limits). Also, the proportion of intercalary rather than terminal vesicles was higher in shock treatments than in fluctuating temperature treatments.

TABLE 2. Effect of temperature treatments on formation of vesicles by germinating *Cronartium ribicola* basidiospores on collodion membranes

Treatment	Total germlings	Vesicles (%)		
		Intercalary	Terminal	Total
16 C constant	26,397	3.6	0.9	4.5
Shock[a]	14,392	9.7	0.4	10.1
Fluctuating[b]	18,220	8.3	7.4	15.7

[a]For temperature-shock experiments spores were cast on membranes and incubated for 16 hours at 16 C, transferred to incubators at 24 or 28 C for 1 or 2 hours, and then returned to 16 C for the remainder of the 72-hour incubation period.

[b]Fluctuating temperatures were tested by incubating spores on membranes in an outdoor mist chamber where temperatures ranged from 16 to 18 C at night up to 27 C during the day, and in a controlled environment growth chamber in which temperatures fluctuated between 7 and 22 C on a 12-hour cycle.

The duration of incubation at 16 C prior to temperature shock affected the number and type of vesicles formed. In these trials spores were incubated for 4, 10, or 16 hours at 16 C, transferred for 1 hour to 28 C, and returned to 16 C. Controls incubated at 16 C yielded 2.7% vesicles, all intercalary. Temperature shock treatments produced more total vesicles than the controls (5.2, 12.3, and 6.4%, respectively), and terminal vesicle production ranged from 96% for the 4-hour preincubation membranes to 19% for the 16-hour preincubation set.

The effect on vesicle formation of changes in the osmotic strength of the solution on which membranes floated also was tested. Spores on membranes were incubated on water for 17 hours at 16 C, and then membranes were transferred to a water control and to 0.05 M, 0.5 M, and 6.0 M glucose solutions. Half the membranes were transferred back to water after 5 hours, and the others remained on the glucose solutions for the remaining 48 hours of incubation. All vesicles observed had infection hyphae. Vesicle formation on the water control membranes was 2.2%. Vesicle formation was significantly increased on the 0.05 M glucose solution with both the 5-hour (4.4%) and the prolonged exposure (4.8%), and on the 0.5 M (15%) and 6.0 M (4.2%) solutions with the 5-hour exposure only. Similar results were obtained when the experiment was repeated with sodium chloride solutions.

Thick germ tube and secondary spore formation.—Under certain moisture conditions, as explained below, basidiospores with thick germ tubes, or with sterigmata and secondary spores (Fig. 1-G), occurred on collodion membranes, glass coverslips, water agar, and *P. strobus* needle surfaces. Thick germ tubes were 3-5 μm in diameter and usually 10-40 μm long, although clumps of spores with thick germ tubes up to 100 μm long were observed. Thick germ tubes were sometimes once or twice branched, and often ended in sterigmata, occasionally with abortive secondary spores attached (Fig. 1-H).

These structures were common only in association with drops of water. On collodion membranes less than 1% of

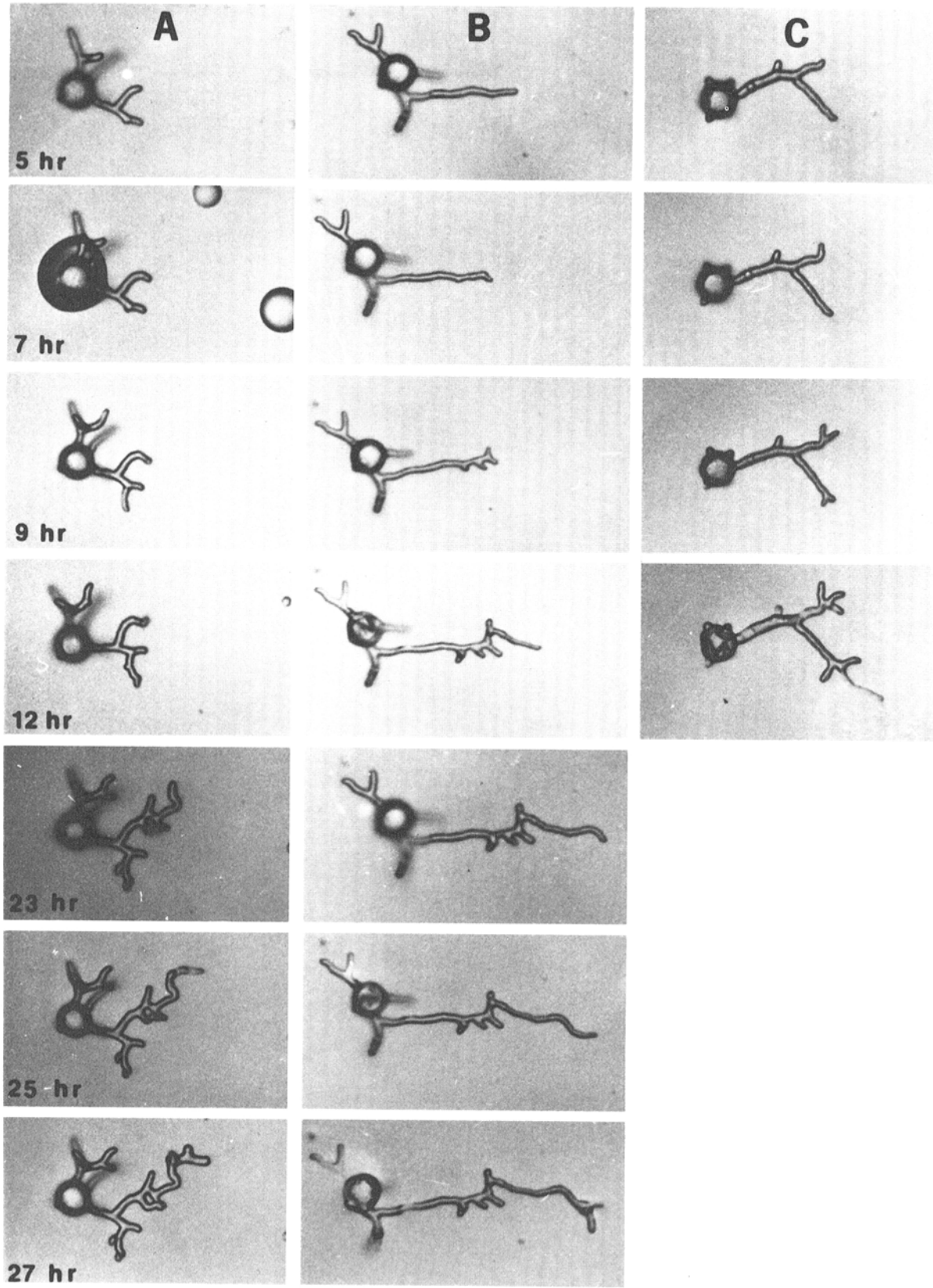

Fig. 3-(A to C). Sequential development of zigzag growth by *Cronartium ribicola* basidiospores cast at a density of approximately 100 spores per mm^2 and incubated on collodion membranes at 16 C for up to 27 hours. **A)** Zigzag pattern that developed from growth of two branches from the dominant germ tube. **B)** The formation of true branches behind the germ tube tip. **C)** A series of finger-like branches that developed as a succession of growing points formed at the tips of the two major branches formed by the dominant germ tube.

the spores germinated and formed secondary spores or thick germ tubes, except when membranes were exposed to water mist, when 20% did so. When spores were cast onto glass coverslips incubated in petri plate moist chambers, and exposed to periodic mist, only 76% of the spores germinated and 41% of germinated spores formed thick germ tubes or sterigmata with or without secondary spores. On unmisted coverslips, 99% of the spores germinated, and only 3% of these formed thick germ tubes or sterigmata. On *P. strobus* needle surfaces where droplets of water accumulated, this type of germination was observed frequently. During incubation of inoculated trees in dew chambers, discrete dew droplets formed along the needles. Spores in contact with these drops clumped together and most formed secondary spores or thick germ tubes, or failed to germinate. In contrast, spores on trees incubated in saturated atmospheres but without formation of visible dew droplets did not clump, and secondary spores and thick germ tubes were infrequent.

The influence of water drops in inducing thick germ tubes or secondary spores appeared to be through the effect on aeration conditions. Spores cast onto a water film were often supported by the surface tension, and if they germinated, they usually formed normal thin germ tubes, often growing into the air away from the water surface. Spores that were suspended but not further agitated settled to the bottom and did not germinate. Spores maintained in suspension by continuous agitation usually germinated to form thick germ tubes, frequently with sterigmata and aborted secondary spores.

Zigzag germ tube growth.—Zigzag germ tubes were characterized by an angular pattern of the main germ tube axis and the presence of numerous short side branches on the germ tube (Fig. 2-C to E). This type of development usually contrasted sharply with the relatively unbranched, straight, or smoothly-curved germ tubes which were considered normal. The occurrence of zigzag germ tubes severely limited study of factors controlling vesicle differentiation, since vesicles did not form on zigzag germ tubes.

Zigzag growth reduced the effective length (straight line distance from spore to furthest germ tube apex) to one-half (75 μm) that of normal germ tubes (150 μm), but the total length (199 μm) (including branches) was not significantly less than in normal germination (219 μm).

Zigzag germ tubes elongated from a succession of growing points, in contrast to continuous elongation of a single growing point exhibited by normal germ tubes. Each succeeding germ tube apex elongated for a short distance and stopped. A new growing point then arose either immediately behind the apex, resulting in a change of direction but no branch, or further back from the old apex, in which case the resulting "branch" was the previous germ tube apex. In addition, some branches arose behind a temporarily arrested apex, which later resumed growth. In some cases where zigzag growth was irregular, growth proceeded alternately from two growing points, resulting in a loosely branched appearance. The sequential development of three germinating spores illustrated in Fig. 3 demonstrates these points. In A both branches of the dominant germ tube elongated initially, with a new growing point originating behind the original tip after growth stopped on the lower branch. The upper

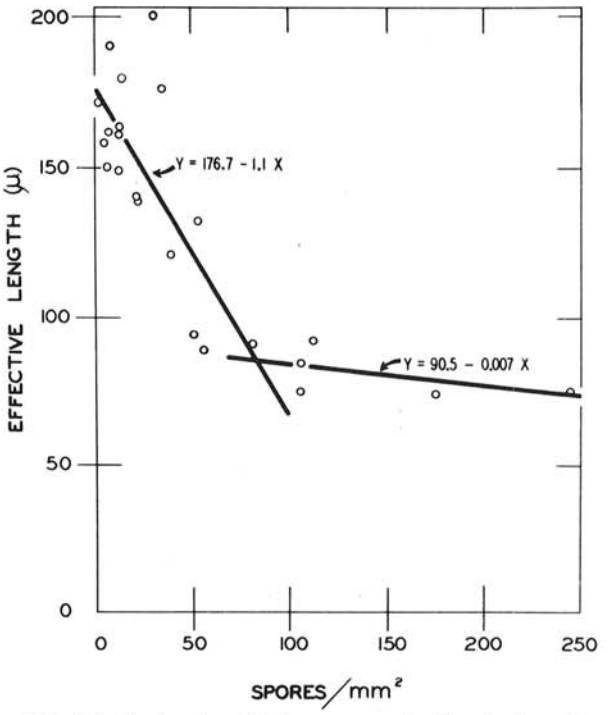

Fig. 4. Reduction in effective germ tube length through development of a zigzag branching pattern by *Cronartium ribicola* basidiospores cast at increasing spore densities on collodion membranes and incubated at 16 C for 72 hours.

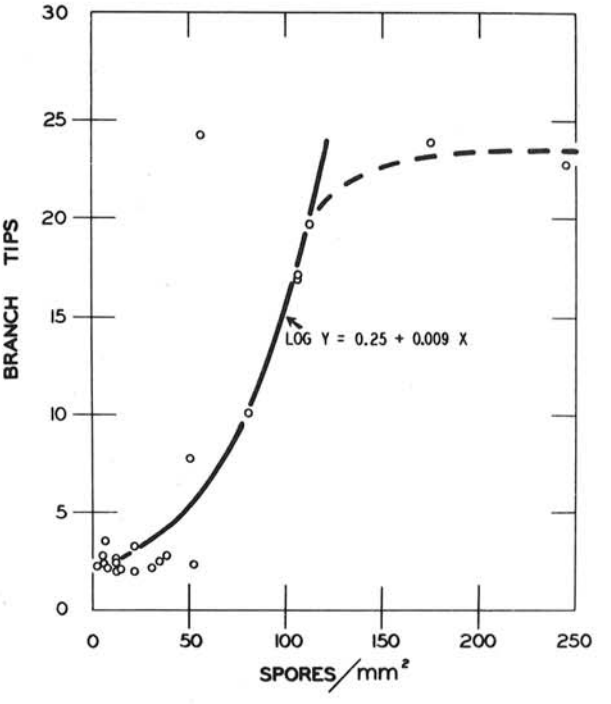

Fig. 5. Increase in number of branch tips on germ tubes through development of a zigzag branching pattern by *Cronartium ribicola* basidiospores cast at increasing spore densities on collodion membranes and incubated at 16 C for 72 hours.

TABLE 3. Induction of zigzag growth by germ tubes from *Cronartium ribicola* basidiospores on low-spore-density collodion membranes positioned above high-spore-density membranes in cover-glass microchambers at 16 C

Treatment	upper membrane / lower membrane		
	spore density (spores/mm^2)	(branch tips per germ tube)	effective length (μm)
low spore density	5	17	92
high spore density	88	22	81
low spore density	4	4	169
empty membrane	0	....	...

branch took over active growth after 12 hours, but after 25 hours a new growing point arose behind the old apex. The spore in B illustrated the formation of true branches behind the germ tube tip, as well as formation of branches resulting in a succession of growing points. The spore in C twice originated a series of finger-like branches as a succession of growing points formed from a single tip.

Factors influencing zigzag growth.—The induction of zigzag growth by a volatile substance associated with the spores was indicated by correlation of zigzag growth with spore density, by induction of zigzag growth across an airspace, and by elimination of the zigzag-growth-inducing stimulus with an airstream.

The correlation between spore density and zigzag growth was determined by measuring the effective germ tube length and number of sequential growing points (branch tips) of germinated spores at each of a series of spore densities. Spore density on collodion membranes was controlled by varying the duration of the spore-cast period. Measurements for twenty-five germinated spores were averaged for each of four membranes at each spore density. The results from four separate trials are summarized in Fig. 4 and 5. In general, the effective length decreased and the number of branch tips increased with increasing spore density. Both length reduction (Fig. 4) and number of growing points (Fig. 5) reached a limit at spore densities between 100 and 150 spores per mm^2.

Induction of zigzag growth across an airspace was demonstrated in modified coverglass microchambers. Membranes with a low spore density (LSD membranes) were mounted on coverglasses and inverted across strips of No. 2 coverglass, over high-spore-density membranes (HSD membranes). As a control, LSD membranes were placed above membranes without spores. After a 72-hour incubation, germ tubes on LSD membranes over HSD membranes were shorter and had more branch tips than those on LSD control membranes, but were longer and less branched than those on the HSD membranes (Table 3). The differences were significant ($P = 0.01$) for each treatment. The experiment was conducted three times with similar results.

The occurrence of the zigzag germination pattern could be affected by passing an airstream over spores germinating on membranes. HSD membranes (average of 115 spores per mm^2) floating in covered or partly uncovered petri dishes were placed in a Plexiglas box through which a humidified airstream was passed. Three trials gave comparable results and the data were pooled. Germinating spores in closed dishes formed zigzag germ tubes which averaged 79 μm in length and produced an average of 19 branch tips. Those in partly uncovered dishes had an average length of 145 μm and an average of five branch tips.

The occurrence of zigzag growth was modified by the nature of the substrate. It frequently occurred on collodion membranes, paraffin-oil collodion membranes, Millipore filters, and on the surfaces of *P. strobus* needles. But germinated spores on glass, waterproof and nonwaterproof cellophane, water agar, and water surfaces were relatively unbranched.

There also seemed to be an unknown factor associated with a certain time of year and which was independent of spore density. In the three years during which germination tests were conducted, uncontrollable zigzag growth occurred primarily during the months of January, February, and March. During these periods, although the degree of zigzag growth increased with increasing spore density, there was considerable branching and reduction in length of germ tubes even at the lowest spore densities.

Nuclear status of normal, zigzag, and differentiated germ tubes.—Comparative observations of nuclei were made after basidiospores had germinated to form normal thin germ tubes, a zigzag pattern of branching, or vesicles. Freshly cast basidiospores were usually uninucleate.

Approximately 60% of normal, thin, unbranched germ tubes were binucleate after an incubation period of 72 hours. Migration of nuclei out of the spore occurred within 4-16 hours after spore deposition. After 72 hours, only 5% of the nuclei of normally germinated spores remained in the spores. Nuclear shape was variable in all types of germ tubes (Fig. 2-D). Many nuclei were drawn out into strands about 40 μm long, and they often extended slightly into branches of the germ tube, or folded back upon themselves. Others were compact and less than 5 μm long.

With zigzag germ tubes, both nuclear division and migration were different from that in normal germ tubes. Most zigzag germ tubes remained uninucleate; in a representative trial in which 150 each of zigzag and normal germ tubes were examined, less than 1% of the zigzag germ tubes and 61% of the normal germ tubes were binucleate. Also, after 72 hours 41% of the nuclei in zigzag germinations remained in the spores, whereas only 5% of the nuclei remained in spores with normal germ tubes. In zigzag germ tubes nuclei were always far behind the site of origin of new growing points; less than 2% of such germ tubes had nuclei in the tip one-third of the germ tube where branches were initiated.

Nuclear division did not necessarily accompany vesicle formation, and germ tube nuclei bore no particular spatial relationship to developing vesicles. As with normal germ tubes, about 60% of differentiated germ tubes were binucleate after 72 hours. At the time of vesicle induction (approximately 16 hours after spore deposition) nuclei were in the basal two-thirds of the germ tube, away from the point of vesicle formation. After 72 hours, they were found with about equal frequency proximal or distal to the vesicles, but seldom within a vesicle (Fig. 1-F).

DISCUSSION.—Since substomatal vesicles are constantly associated with needle infection of white pines

by *C. ribicola,* and since such infection structures were demonstrated as being derived only by the differentiation of typical long, thin, relatively unbranched germ tubes, the germination of basidiospores to form such germ tubes was considered to be the typical or normal type of development that leads to needle infection. The formation of secondary basidiospores or a zigzag type of germ tube is the result of various influences that are ultimately unfavorable for needle infection.

Vesicle formation.—Interest in factors which influence vesicle formation was considered of importance from the viewpoint of understanding the infection process. The stimulation of vesicle formation by a variety of shock treatments suggests that these swellings form as a result of a temporary disruption of germ tube elongation. If the disrupting factor is removed, or the germ tube equilibrates while its apex is still able to resume growth, then a vesicle with infection hypha is formed. A similar pattern of shock-induced swelling followed by normal growth was reported by Robertson (40, 41) for *Fusarium* spp. and other hyphomycetous fungi. With *Puccinia graminis* f. sp. *tritici* reduced carbon dioxide or increased temperatures were required for vesicle formation (43, 46, 53). Dickinson (9) postulated that the pattern of germ tube growth, including zigzag growth and infection structure formation, is determined by the frequency and height of ridges on the substrate surface. *Cronartium ribicola* basidiospores, however, commonly produced vesicles and zigzag branching on germ tubes growing completely away from the substrate. These observations, together with the reported differentiation of germinating urediospores in shake culture (13), suggest that factors other than substrate geometry may determine the pattern of germ tube development.

The possible function of infection structures is a matter of recent discussion in the literature, especially relative to the artificial culture of several rust species from urediospores. Differentiation is apparently not a prerequisite to saprophytic growth (44, 50). Vesicle formation may be necessary for establishment of the dikaryotic state (49), although dikaryotic saprophytic mycelia have also been reported from urediospores without infection structure differentiation (3). Basidiospores do not form a dikaryotic mycelium, so such a role is not possible. In addition, infection structure formation has been correlated with activation of RNA and protein synthesis (14, 39); but, again, reports of saprophytic growth without infection structure formation suggest that synthesis can be initiated without these structures. Although nuclear division in germinating urediospores of several rust species occurs on membranes only with differentiation of infection structures (8, 31), that is not true for germinating basidiospores of *C. ribicola*. Finally, although conditions for differentiation of infection structures appear to be essential for the infection of host plants as well as tissue cultures by urediospores (4, 8, 31), such may not be the case for basidiospores of *C. ribicola* (18).

The experimental results reported here, together with the observations of vesicles on penetrating hyphae of nonrust fungi of a wide range of parasitic capabilities (15, 25, 34, 52), have suggested the hypothesis that vesicles are not an essential part of the infection process, but rather a result of disrupted growth following a change in the germ tube environment. With *C. ribicola* the same conditions shown to stimulate vesicle formation on artificial substrates might be encountered by a penetrating basidiospore germ tube. Perhaps vesicle formation might best be considered as a programmed response to a number of stimuli.

Secondary spore formation.—Secondary spore formation appeared to be a special type of germination resulting from excess water around the spore. Contrary to the reports of Bega (2) and Hirt (21) the frequency of secondary spore formation was unaffected by temperature.

Secondary spore formation provides basidiospores with a means of redistribution from unfavorable environments (26). It does not appear to be stimulated by nonhost substances, but rather by unfavorable micromoisture conditions on any substrate. The observed differences in frequency of secondary spore formation on various host and nonhost substrates (28, 42) may reflect differences in surface wettability and available moisture rather than a chemical distinction between host and nonhost substrates.

Zigzag growth.—Zigzag growth from basidiospores has not been reported previously. Time-lapse photography of *Puccinia coronata* germinating urediospores by Dickinson (10), revealed zigzag growth proceeding from a succession of growing points, as described here for *C. ribicola* basidiospores. Dickinson felt that zigzag growth occurred in response to a particular height and frequency of oriented ridges in the substrate, and resulted in directed growth perpendicular to those ridges. Lewis and Day (29) published photographs of germinated urediospores whose growth was apparently oriented thigmotropically by the crystalline lattice of wax on the surface of wheat leaf cuticle. On damaged leaf cuticle, however, germ tubes had typical zigzag features and showed no evidence of directed growth. These differences suggested that zigzag growth and directed growth occur in response to different stimuli. The observed growth of zigzag germ tubes of *C. ribicola* completely away from the substrate also indicates that factors other than thigmotropic stimuli can trigger zigzag growth.

Our experiments indicated that a volatile compound associated with germinating basidiospores induced zigzag germ tube growth. The failure of germinated spores at high densities on water or agar to form zigzag germ tubes may be explained by absorption or dilution of the factor as it was released. Although spore densities on inoculated needles were often much higher than on membranes, zigzag growth occurred less regularly. This may have been due to the presence of dew on the needles and to the greater amount of air movement around the inoculated seedlings than over membranes in closed petri dishes.

During certain periods zigzag growth occurred at even the lowest spore densities, indicating some external controlling factor. Several workers have reported similar periods of abnormal germination by cereal rusts, which they were able to correlate with atmospheric conditions (19, 45).

Zigzag germ tube growth was an important factor in this study because it prevented vesicle formation. Zigzag growth could also be of significance in the infection process under conditions that favored this type of

development. The reduction in effective germ tube length and the inhibition of nuclear movement and division which accompany zigzag growth might reduce a germ tube's chances of reaching a stoma or of continuing development within the host needle.

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