Cold Inactivation of *Phytophthora cinnamomi*

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ABSTRACT

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Mycelium of *Phytophthora cinnamomi* in cornmeal agar and chlamydospores in naturally infested soil were inactivated on a thermal gradient plate at temperatures below 0 C. Rate of inactivation was directly related to number of degrees below 0 C. Inactivation of mycelium of *P. cinnamomi* occurred in 2, 6, or 16 days, at −6.7, −3.8, and 1.4 C, respectively. In a sandy-loam soil, chlamydospores of *P. cinnamomi* were inactivated after 2, 17, or 29 days at −6.4, −3.4, and −0.5 C, respectively. Acclimatization of agar cultures or infested soil for 5–7 days at 4 C did not increase tolerance of *P. cinnamomi* to subzero temperatures. Inoculum of *P. cinnamomi* in infested root segments of *Abies fraseri*, in colonized oat grains, or in naturally infested soil, was inactivated during winters (1976–1977 and 1977–1978) when soil temperature at a depth of 10 cm dropped to 0 C or below, but not during the winter of 1975–1976 when soil temperatures remained above 0 C.

Phytophthora cinnamomi Rands causes a serious root rot of ornamentals and other crops throughout the subtropical and temperate regions of the world (13). Although the pathogen can survive in moist soil for at least 6 yr at 20 C in the absence of a host (14), inactivation of inoculum by soil temperatures below 0 C may prevent overwintering in northern areas of the temperate region. In naturally infested soils in New Jersey (12) and Berlin, West Germany (8), *P. cinnamomi* did not survive winter conditions as measured by host infection assays. Steekelenburg (11) found that *P. cinnamomi* survived 12 but not 20 days at −2 C as indicated by a host infection test on cuttings of *Chamaecyparis lawsoniana* (A. Murr.) Parl. 'Ellwoodii.'

Infection and survival experiments with *P. cinnamomi* root rot of azalea at our research nursery in Raleigh, NC, suggested that cold weather may have an adverse effect on survival of the pathogen in soil and host tissues (1).

The present study describes further experiments in which a constant-temperature, thermal gradient plate was used to measure inactivation under controlled conditions as well as field experiments at the nursery.

MATERIALS AND METHODS

Test fungus. *P. cinnamomi* isolate 101 from diseased roots of *Rhododendron* sp. was used to study inactivation in agar culture. Three-day-old cultures of *P. cinnamomi* grown on cornmeal agar (CMA) at 25 C in 50-mm-diameter plastic petri dishes were precut with a sterile, 4-mm-diameter cork borer at the margin of the colony for later sampling. Colonies of *P. cinnamomi* at 3 days contained only mycelium.

Soils. Two naturally infested soils (one a sandy loam [pH 5.6, measured in a 1:2 soil to water mixture] from an *Abies fraseri* (Pursh) Poir nursery bed in western North Carolina and the other a clay soil (pH 5.8) from an azalea [*Rhododendron obtusum* Planch.] bed in Raleigh, NC) were used to study inactivation of *P. cinnamomi* in soil. The soils were collected in May from areas in nursery beds where the plants had developed symptoms of Phytophthora root rot. Soils were held at ambient room temperature (20–22 C) for 1–5 wk in plastic bags prior to use. Soil moisture was 20% and 25–30% for the sandy-loam soil and clay soil (multiple collection), respectively.

Thermal gradient plate. Cold inactivation of *P. cinnamomi* was studied by using a thermal gradient plate (TGP) similar in principle to one described by Rowe and Powelson (7). The TGP consisted of a square aluminum plate (2 × 70 × 70 cm) divided into 100 grids (6.4 × 6.4 cm) arranged 10 grids by 10 grids. Refrigerated water from a constant-temperature reservoir was circulated along one edge and heated water from a second reservoir was circulated along the opposite edge, which established an approximately 12 C temperature gradient across the plate. A 10-cm-high wooden frame covered with a heavy plastic film was placed over the TGP to minimize ambient temperature effects. The TGP was maintained in a 5 ± 2 C cold room during experiments.

Cold inactivation. Petri dishes containing either CMA cultures of *P. cinnamomi* or approximately 16 g of infested soil were arranged across the TGP in five alternate rows with 10 dishes per row. Thus, dishes in each row were incubated at a different constant temperature. Temperature readings were taken daily on adjacent rows using a mercury thermometer embedded horizontally through the side of a petri dish containing CMA or soil. Temperature variation within a row was usually less than ± 0.5 C. Temperature at which test dishes were exposed was estimated by interpolation of data from linear regression analysis for the five rows actually measured.

Agar disks or soil samples were removed to determine inactivation at various intervals after incubation on the TGP. One agar disk was removed from five dishes at each temperature and transferred to fresh CMA. Inactivation was determined by counting the number of agar disks from which *P. cinnamomi* did not grow after 5 days at 25 C. Inactivation was calculated as the inverse of percent survival at each sampling interval. Experiments were repeated three times.

A combination of wet sieving and a selective medium (PCH) was used to assess inoculum density of *P. cinnamomi* in soil (10), which exists primarily as chlamydospores (4,9). At each sampling interval one dish at each constant temperature was removed from the TGP, and three 5-g subsamples were assayed. Additional dishes were sampled to determine soil moisture. Inactivation at the various temperatures was determined by comparing inoculum density over time with the initial inoculum density. Experiments were repeated three times.

Field experiments at the research nursery. In the winter of 1975–1976, 1-cm-long root segments infected with *P. cinnamomi* isolate 103 were collected from *A. fraseri* seedlings in the greenhouse and used to infest the sandy loam soil (collected from a disease-free area of the *A. fraseri* nursery) at the rate of 50 root

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segments per 400 cm^3 of soil. The infested soil was placed in 10-cm-diameter clay pots that were buried in natural soil to the top edge of the pot. Root segments were screened from randomly selected pots at approximately 2- to 4-wk intervals and divided into five lots of 10 segments each. Root segments were surface disinfected for 1.5 min in 0.5% NaOCl, rinsed three times in distilled water, and cultured for *P. cinnamomi* on a modified pimaricin-pencillin-polymyxin medium (2) containing 10 mg pimaricin per liter.

In the winter of 1976–1977, survival of *P. cinnamomi* in infected roots and naturally infested sandy loam soil was studied in two experiments. Infected root segments were collected and used to infest both the sandy loam soil and clay soil. Naturally infested soil was buried in pots as had been the soils containing root segments. Inoculum density was monitored in the naturally infested soil at 2- to 4-wk intervals. A 1-ml sample of soil dilution (10 g soil in 100 ml H_2O) was spread on the agar surface of each of 10 petri dishes containing modified Kerr's medium (5). After 2 days of incubation in the dark at 20 C, the soil film was washed gently from the petri dishes and the subsurface colonies of *P. cinnamomi* were counted microscopically. Three 10-g subsamples were assayed for each sampling period. Ten additional 1-ml soil dilution aliquots were air-dried to determine the actual amount of soil transferred to each dilution plate for correction to propagules per gram of soil.

In the winter of 1977–1978, oat grains infested with six isolates of *P. cinnamomi* were incorporated (90 grains per 400 cm^3) into either a peat moss-sand-sterile soil medium (1:1:1, v/v), a pinebark-sand medium (1:1, v/v), a pinebark medium, or a pinebark-sand-peat medium (6:3:1, v/v). Ten-centimeter-diameter pots containing the infested media were placed in wooden flats on the surface of a pinebark-covered container area in a lathhouse at the nursery. Oat grains were recovered from pots at monthly intervals and assayed for *P. cinnamomi* as described above for root segments.

Soil temperature data (10-cm depth, bare ground) for a monitoring site 16 km from the nursery were taken from monthly National Weather Service records.

RESULTS

Cold inactivation in agar culture. Inactivation of *P. cinnamomi* in CMA disks was directly related to temperature below 0 C (Fig. 1). At the coldest temperature (−6.7 C) agar disks were frozen after a few hours on the TGP. At temperatures above −6.7 C agar disks did not freeze. Inactivation occurred within 2, 6, or 16 days, at −6.7, −3.8, and −1.4 C, respectively (Fig. 1). At temperatures above 0 C survival of *P. cinnamomi* extended to 35 days when the experiment was terminated due to desiccation of the CMA.

In a second experiment, 2- or 3-day-old cultures of *P. cinnamomi* in CMA were acclimated at 4 C for 5 days prior to placement on the TGP. Rate of inactivation of *P. cinnamomi* disks that were acclimatized was similar to nonacclimatized disks. For instance, at −7.5 and −3.5 C only 8 and 48 hr, respectively, were required for 100% inactivation of acclimatized disks.

Cold inactivation in soil. Inactivation of *P. cinnamomi* in naturally infested soil also was directly related to temperature; chlamydospores in soil were inactivated at about the same rate as mycelium in agar. In the sandy loam soil, inactivation occurred within 2, 17, or 29 days at −6.4, −3.4, and −0.5 C, respectively (Fig. 2A). At 2.5 and 5.5 C, inoculum density of *P. cinnamomi* declined, but some propagules were viable up to 58 days. In the clay soil, initial inoculum density was half the initial inoculum density of the sandy loam soil. However, the pattern of inactivation of *P. cinnamomi* was similar to that in sandy loam soil. For example, at −5.8, −3.2, and −0.5 C inactivation of *P. cinnamomi* occurred within 1, 6, and 16 days, respectively (Fig. 2B). At 2.2 and 4.9 C, *P. cinnamomi* persisted until the experiment was terminated at 44 days.

The sandy loam soil was acclimatized at 4 C for 7 days prior to placement on the TGP in another experiment. Inoculum density dropped from an initial 9.1 propagules per gram of soil to undetectable levels within 24 hr at −5.2 C. At −2.2 C inoculum density of *P. cinnamomi* was unchanged after 2 days, but changes in inoculum density in soil usually occurred over a longer period

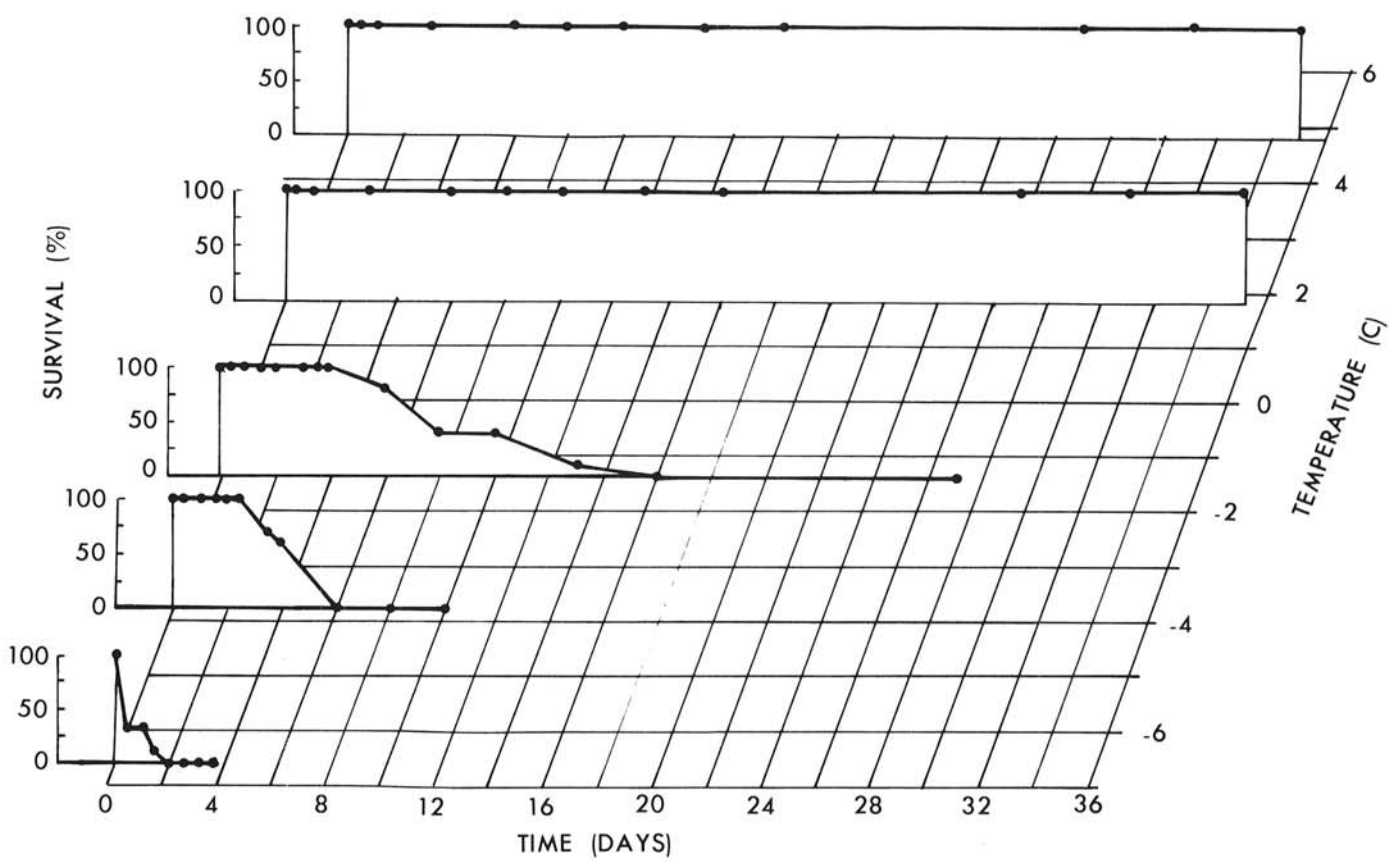

Fig. 1. Survival of *Phytophthora cinnamomi* in cornmeal agar disks during 36 days on a constant-temperature, thermal gradient plate.

than did inactivation in agar disks.

Cold inactivation of inoculum in overwintered soils. Survival of *P. cinnamomi* in *A. fraseri* root segments was apparent over the winter of 1975–1976. Rate of survival, however, fell from 78% recovery in October to 30% recovery in late March with the greatest single-month decline between October and November (Fig. 3). Soil temperature at 10 cm averaged over 4.5 C (readings taken at 0700 hours) during the winter, and 1.5 C was the lowest recorded temperature.

During the winter of 1976–1977, *P. cinnamomi* was inactivated in root segments buried in either the sandy loam or clay soil after 20 days. Initially, root segment colonization of *P. cinnamomi* was 36%. The experiment was started 5 January 1977. During the six sampling periods over 2.5 mo, *P. cinnamomi* was recovered at the initial level within the first week, but recovery dropped to 0% in four of the next five periods with only a 3% recovery in one of the five sampling periods for each soil type.

Inoculum density of *P. cinnamomi* in the naturally infested, sandy loam soil decreased from 7.3 propagules per gram of soil in November 1976 to an undetectable density in February 1977 (Fig. 4). Additional soil assays in late February and March also failed to detect the fungus. Soil temperatures at 0700 hours in January 1977 were at or below 0 C for 14 days. The lowest reading reported was −1 C. In February 1977, 3 days had temperature at 0 C.

During the winter of 1977–1978, *P. cinnamomi* in infested oat grains in the various media was inactivated by February 1978. No difference in rate of inactivation was found among the media (Fig. 5). Media were frozen most of the winter. There were 6, 17, 25, 23, 11, and 0 days with air temperatures at or below 0 C during the months of November, December, January, February, March, and April, respectively. In late April, however, 1% of the oat grains contained viable *P. cinnamomi* in the bark and bark-sand-peat media.

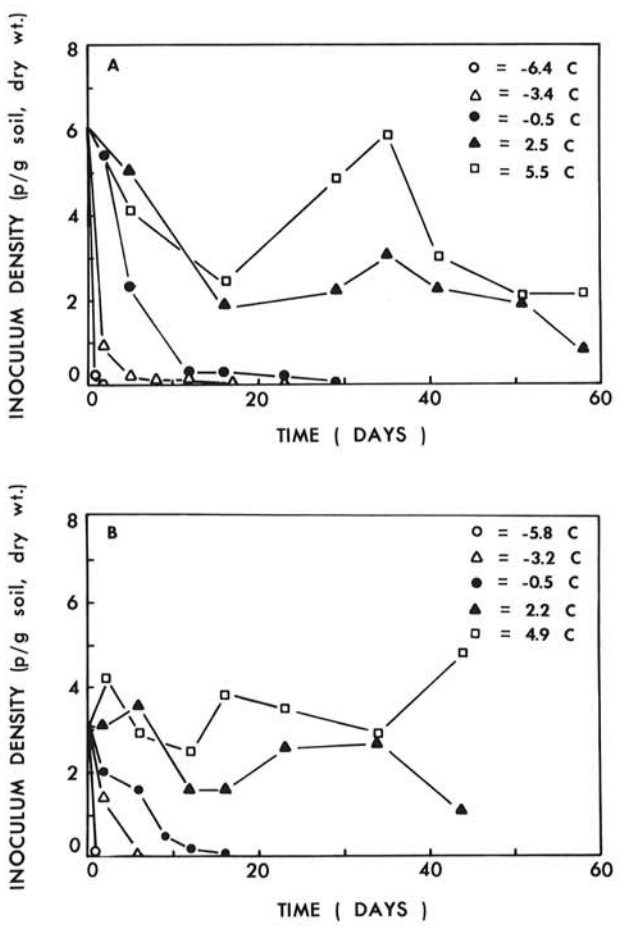

Fig. 2. Cold inactivation of *Phytophthora cinnamomi* in two naturally infested soils, **A,** sandy loam soil, and **B,** clay soil, exposed to different constant temperatures on a thermal gradient plate. Inactivation calculated from a comparison of initial inoculum density to inoculum density at subsequent sampling periods.

DISCUSSION

Cold inactivation of *P. cinnamomi* is not a new concept, but as Zentmyer (13) pointed out, little information is available on the effect of cold temperature on this pathogen. Quantitative data, not previously available, on the relationship of constant cold temperature to inactivation of *P. cinnamomi* were obtained in the laboratory with the TGP. In addition, field studies on overwintering of inoculum confirmed the laboratory observations.

A temperature of −6 C inactivated *P. cinnamomi* from CMA disks and naturally infested soil in only 2 days on the TGP. At temperatures below 0 C, survival of the fungus increased but inactivation eventually occurred. At temperatures above 0 C inactivation did not occur. Sauthoff (8) reported that 4 wk at −6 C were required for inactivation of *P. cinnamomi*.

Apparently, mycelium and chlamydospores of *P. cinnamomi* are equally sensitive to cold inactivation since CMA disks with mycelium and naturally infested soil with chlamydospores were

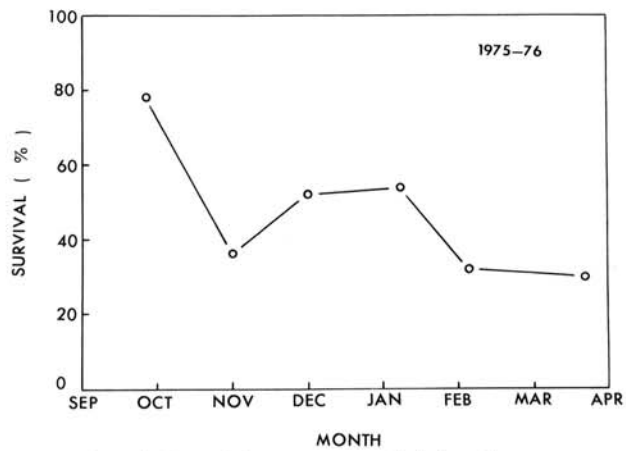

Fig. 3. Survival of *Phytophthora cinnamomi* in infected root segments of *Abies fraseri* incorporated in a sandy loam soil and buried outdoors in 10-cm-diameter pots during the winter of 1975–1976.

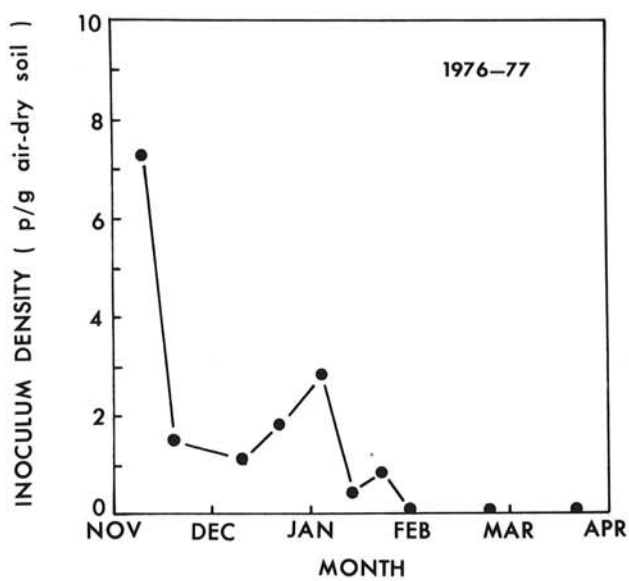

Fig. 4. Cold inactivation of *Phytophthora cinnamomi* in a naturally infested, sandy loam soil buried outdoors in 10-cm-diameter pots during the winter of 1976–1977. Inactivation calculated from a comparison of initial inoculum density to inoculum density at subsequent sampling periods.

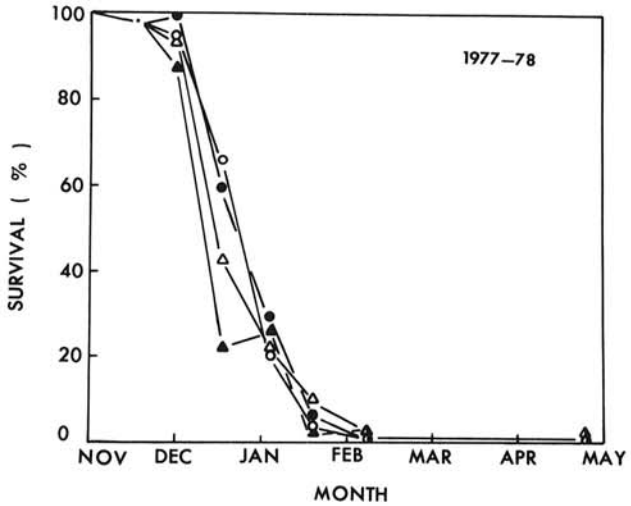

Fig. 5. Cold inactivation of *Phytophthora cinnamomi* on infested oat grains incorporated into a peat-sand-sterile soil medium (o), a pinebark-sand medium (•), a pinebark medium (△), or a pinebark-sand-peat medium (▲), and placed in wooden flats on the surface of a container-storage area under a lathhouse during the winter of 1977–1978. Inactivation calculated as the inverse of percent survival.

inactivated at about the same rate. The thick-walled nature of naturally produced chlamydospores (9) did not enhance chlamydospore survival in soil under conditions on the TGP.

Research workers have studied cold inactivation of *P. cinnamomi* by using host infection assays of infested soil that was overwintered outdoors. For instance, White (12) in 1936 found that soil infested with *P. cinnamomi* that was exposed to winter temperatures in New Jersey was not infective to rhododendron seedlings the following spring. Soil temperatures at 10 cm were below −1.1 C occasionally during the winter with a minimum of −3.9 C for 5 hr. Sauthoff (8), in Berlin, used apple baits and *Erica gracilis* L. to monitor the presence of *P. cinnamomi* in soil overwintered outdoors. None of the 50 plants developed root rot when planted into the soil in the spring, but in similar soil kept in the greenhouse during the winter, all 50 plants were diseased after planting in the spring. Soil temperatures at which *P. cinnamomi* was inactivated were not given. Steekelenburg (11) reported that *P. cinnamomi* survived in naturally infested soil overwintered in Boskoop, The Netherlands, during the winters of 1970–1971 and 1971–1972. Soil temperatures at 10 cm were never below 0 C. Krober (6) in Berlin, Germany, reported that during a 6-yr study *P. cinnamomi* in artificially infested soil placed in pots survived outdoor winter conditions when air temperatures were mild with few days below 0 C as measured by direct plating and apple baiting techniques.

In the present study, *P. cinnamomi* survived the winter of 1975–1976 in infected root segments. Soil temperatures, however, did not fall below 0 C. During the winters of 1976–1977 and 1977–1978 when soil temperatures were at or below 0 C during a portion of several months, *P. cinnamomi* was inactivated in infected root segments, infested oat grains, and two different naturally infested soil types.

Acclimatization of CMA cultures or naturally infested soil for 5–7 days at 4 C prior to placing on the TGP did not enhance survival of *P. cinnamomi* at temperatures below 0 C. Gerlach et al (3) reported a highly beneficial effect on survival by acclimatizing cultures of *P. citrophthora* at 4 C for 4 days prior to subjecting cultures to −21 C. Cultures not acclimatized at 4 C failed to survive at −21 or −31 C. Differences in effects of acclimatization on *P. cinnamomi* and *P. citrophthora* and the general lack of survival of *P. cinnamomi* at temperatures below 0 C may explain, in part, the geographical distribution of this pathogen.

Although cold inactivation of *P. cinnamomi* may significantly lower the inoculum density of the pathogen in soil, propagules of *P. cinnamomi* may escape cold inactivation in nature by distribution soil depths not exposed to lethal temperatures as described by Steekelenburg (11). Propagules of *P. cinnamomi* in large, infected roots or stems and debris (5–10 mm in diameter) in soil may not be subject to inactivation at the same rate as in roots used in this study (1–2 mm in diameter). In epidemiological terms, the effect of inactivating 99% of the inoculum by cold temperatures may not affect disease development if 1% of the original inoculum is sufficient to incite 100% disease.

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