Role of Primary and Secondary Zoospores of *Plasmodiophora brassicae* in the Development of Clubroot in Chinese Cabbage

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ABSTRACT

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Whether the development of clubroot symptoms is the result of primary (I°) or secondary (II°) zoospore infection has long been debated. By controlling the soil water matric potential with tension plates, soil-membrane column units, and a discontinuous soil cylinder system, infection processes of I° and II° zoospores were interrupted at different times after transplanting 1-day-old Chinese cabbage seedlings into soil infested with 10^7–10^8 spores of *Plasmodiophora brassicae* per gram of dry soil. Root hairs were infected by I° zoospores within 1 day and mature sporangia appeared after 2 days. Active II° zoospores were not observed until the 3rd day, which coincided with the initiation of cortical infection. Within 3 hr of II° zoospore release, cortical infection had occurred. These observations support the necessity of II° zoospores for cortical infection.

Additional key words: soil water matric potential.

The life cycle of *Plasmodiophora brassicae* Wor. is not fully understood, but can be characterized by two phases: the root-hair phase, following root-hair infection, and the cortex/stele phase, following cortical infection (5). A widely accepted life cycle has been proposed by Ingram and Tommerup (4). In this cycle, primary (I°) zoospores infect root hairs and sporangia are formed. Secondary (II°) zoospores produced from these sporangia either reinfect root hairs or fuse in pairs and infect cortical cells; the latter leads to the formation of clubbed roots. Although a number of clubroot workers claim that II° zoospore fusion is required for cortical infection, no one has observed the fusion process (2,4). Similarly, there is no evidence to discount the possibility that I° zoospores, single or fused, can cause cortical infection, except that it has never been observed. Aist (1) pointed out the need to more fully define these points of the life cycle.

Work by Dobson et al (3) showed that root-hair infection can occur at lower soil water matric potentials (ψ_m) than cortical infection. In a silt loam soil, root-hair infection occurred at ψ_m of 0 to −800 mbars, while cortical infection occurred only in soil wetter than −150 mbars. This provides evidence that the two phases are distinct. The authors proposed that the higher ψ_m requirement for cortical infection might reflect the needs of larger zoospores, a result of zoospore fusion. However, it was not determined which types of zoospores, I° or II°, were involved. Consequently, this study was initiated to determine whether I° or II° zoospores give rise to cortical infection.

MATERIALS AND METHODS

A Puyallup Sultan silt loam soil from western Washington with a $CaCl_2$ pH of 5.8, artificially infested with spores of *P. brassicae* at 10^7–10^8 spores per gram (dry weight basis) of soil, was used in each method. Tension plates and membrane systems were set up as described previously (3). Both were maintained at 20–25 C and planted with Chinese cabbage (*Brassica campestris* L. s. sp. *pekinensis* (Lour.) Olsson.) seedlings germinated for 24 hr. Experiments using a completely randomized design were repeated at least once. Several different methods were used to determine the time required for root-hair and cortical infection.

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Method 1: Seedlings started in tension plate funnels at a uniform planting date to determine time required for root-hair and cortical infection. Twenty-one Büchner funnels with fritted glass disks were used as tension plates. Artificially infested soil was placed in each funnel. The funnels were equilibrated to −20 mbars (a ψ_m favorable for cortical infection) for 12 hr, then eight cabbage seedlings were planted in each. At 20 min, and at 1, 2, 3, 4, and 5 days after planting, three funnels were equilibrated to −160 mbars by dropping the water reservoir from 20 to 160 cm below the tension plates to stop further cortical infection. Three funnels were maintained at −20 mbars as controls. Immediately prior to equilibration at −160 mbars, four of the eight seedlings were removed from each of the three funnels. The roots were washed, mounted in water, and observed under a compound microscope for immature and mature root-hair sporangia and II° zoospores. Mature sporangia were distinctly differentiated within the sporangiosori, while immature sporangia were not. Mature sporangia were expressed as a percentage of the number counted on control plants after 5 days, thus generating the data presented in columns one and two of Table 1. When more than ten II° zoospores were observed within 5 min of washing the roots, II° zoospores were scored as positive, generating data presented in column three of Table 1. The four plants remaining in the funnels were allowed to

TABLE 1. Progression of the life cycle of *Plasmodiophora brassicae* after different incubation periods under favorable conditions of high soil moisture

	Life cycle stages			
Time after transplanting	Root-hair infection	Mature sporangia[x]	II° Zoospores present	Plants with cortical infection[y]
20 min	−	0 a[z]	−	0 a[z]
1 day	+	0 a	−	0 a
2 days	+	10 a	−	0 a
3 days	+	58 b	+	53 b
4 days	+	85 c	+	98 c
5 days	+	100 c	+	98 c

[x] Percentage based on the total number observed in control plants after 5 days.
[y] Cortical infection determined by club formation after 6 wk.
[z] Numbers followed by the same letter in each column are not significantly different according to Duncan's multiple range test ($P = 0.05$).

grow for 6 wk and checked for clubroot symptoms, which were assumed to be evidence of cortical infection and were used to obtain the data presented in the last column of Table 1.

Method 2: Seedlings started in tension plate funnels at different planting dates to determine time required for cortical infection. The objectives of using this method for determining requirements for cortical infection were the same as those for method 1, but this method eliminated any variation due to individual funnels. Four tension plates were established at −20 mbars as described above. Three seedlings were planted in each funnel on 6 consecutive days. Twenty minutes after the final planting, the funnels were equilibrated to −160 mbars to stop further cortical infection. Thus, in each funnel a set of three plants were in wet soil (−20 mbars, which is conducive to cortical infection) for 20 min and 1, 2, 3, 4, and 5 days. Root-hair infection was not assessed. Clubroot symptoms, which were assumed to be evidence of cortical infection, were recorded after 6 wk and were used to obtain the data presented in the last column of Table 1.

Method 3: Seedlings started in a soil-membrane column system to determine time required for root-hair and cortical infection. This method duplicated the objectives of Method 1, but used a different method of controlling the soil water matric potential. The method was similar to that described by Dobson et al (3), but without polyethylene glycol. Infested soil was carefully pressed into the open end of a 50-mm-diameter cylindrical semipermeable dialysis membrane that had been tied tightly at one end. The soil-membrane column was about 5 cm long and was placed in a 100-ml beaker filled with water. The purpose of the membranes was to hold the soil as a unit and to allow water to penetrate the soil. Twenty-four soil-membrane columns were prepared and equilibrated for 12 hr. Four seedlings were then transplanted into the saturated soil in each unit. Sets of four soil-membrane units were removed from the water after 20 min, and 1, 2, 3, 4, and 5 days. The plants from one of the four soil/seedling units in each set were observed for mature root-hair infection at these times. Percent mature root-hair infection was based on the 5-day saturation count, giving the data presented in the second column of Table 1. The remaining sets of three membranes were rapidly dried to about −300 mbars as measured by a tensiometer to prevent further cortical infection (3). This was done by removing the membranes and covering the soil/seedling units with dry soil for 0.5 hr.

These units were transplanted into discontinuous soil cylinder systems (3) in the glasshouse where the ψ_m was maintained below −200 mbars. These cylinders, 10 cm in diameter and 20 cm high, were placed in shallow watering pans and filled to about 10 cm from the top with a packed, well-moistened (but not saturated) soil. A 2-cm layer of moist sand was then added. On top of the sand more soil premoistened to −200 mbars was added. The soil/seedling units were transplanted into this top layer of soil. Water was added only to the watering pans in quantities that did not allow the bottom soil to become saturated. Under these conditions, the top layer of soil slowly dried and the ψ_m was never greater than −200 mbars. There was, however, sufficient moisture for root growth to penetrate through the sand to the moist layer below and sustain growth over the 6-wk period. If the bottom soil became saturated, moisture crossed the sand barrier and saturated the top soil, allowing further cortical infection, thus invalidating the experiment. The soil/seedling units transplanted into these cylinders were maintained for 6 wk at which time clubroot symptoms, as evidence of cortical infection, were rated only in the top layer of soil, giving the data reported in the last column of Table 1.

Method 4: Seedlings started in a soil-membrane column system to determine minimum time required for early root-hair infection. Although root-hair infection could be determined by observing morphologically distinct root-hair sporangia, these did not become visible until the second day. To determine if root-hair infection occurred before this time, soil-membrane units with four plants each were equilibrated in water for 20 min and 1 and 2 days. The units were then removed as in method 3 and rapidly dried to about −1,000 mbars to prevent further root-hair infection (3) by covering them with dry soil for 2 hr. The plants were then maintained at −1,000 mbars for 3 days to allow root-hair sporangia to mature. The plants were then washed and observed for evidence of root-hair infection, providing data presented in the first column of Table 1.

Method 5: Seedlings started in a soil-membrane column system to determine the time required for cortical infection after release of II° zoospores. To determine the minimum time required for cortical infection once root-hair sporangia were mature and II° zoospores were released, 20 soil units were used as described above except that the membranes were replaced with paper towels cut and stapled into the same shape. The use of paper towels in place of the dialysis membrane greatly reduced the cost and performed equally well (only when polyethylene glycol is used to create a ψ_m are semipermeable membranes essential). The soil units were equilibrated in water for 12 hr to a ψ_m of approximately 0 bars and planted with four seedlings. The units were maintained in water for no longer than 2 days to ensure that no cortical infection could occur (cortical infection occurs either on or after 3 days). The paper towel units were then taken out of the water, the paper towels were removed, and the plant-soil units rapidly dried by covering them with dry soil for 0.5 hr, which lowered the ψ_m to about −300 mbars. The plant-soil units were then transplanted into soil with a ψ_m between −200 and −300 mbars (which halted cortical infection [3]) for at least 2 days while allowing the maturation of root-hair sporangia. After these 2–3 days, one or two units were removed and the roots were observed for emerging II° zoospores. Only when II° zoospores were observed were the other units rewrapped in paper towels and again placed in water for 20 min, and 3, 6, 12, and 24 hr. After each time period, three soil units were dried rapidly to about −300 mbars to again prevent further cortical infection. These were then transplanted into discontinuous soil cylinders in the glasshouse as in Method 3. Clubroot data as evidence of cortical infection were recorded after 6 wk.

RESULTS

Most of the results of the experiments are summarized in Table 1. Under favorable conditions of high soil moisture, the time required for root-hair infection, mature sporangia development, II° zoospore release, and for cortical infection followed a stepwise progression (Table 1).

Root-hair infection was determined to occur after 1 and 2 days by using Method 4, which prevented further root-hair infection by dropping ψ_m to −1,000 mbars after each test period. Root-hair infection was also observed at 3 days and later in Methods 1 (tension plates) and 3 (membranes). Percent mature sporangia reported in Table 1 were averaged from Methods 1 (tension plates) and 3 (membranes). Mature root-hair sporangia, although present at 2 days, increased significantly between 2–3 and between 3–4 days.

Release of II° zoospores from root-hair sporangia was observed by the third day in Methods 1 (tension plates) and 3 (membranes) (Table 1).

The time necessary for cortical infection, determined by the development of clubroot symptoms, was determined to occur after 3 days by using Methods 1 and 2 (tension plates) and 3 (membranes) by dropping the ψ_m to a range of −200 to −300 mbars, which precluded further cortical infection after each test period. A significant increase in cortical infection that occurred between days 3 and 4 coincided with the release of II° zoospores and a significant increase in percentage of mature root-hair sporangia.

The time required by II° zoospores for cortical infection was 3 hr, and was determined by Method 5 in which paper membrane units, after root-hair infection, were held at −200 to −300 mbars to preclude cortical infection before and after exposure to II° zoospores. Exposure times to II° zoospores were 0.3, 3, 6, 12, and 24 hr. The percentage of plants showing clubroot symptoms after each exposure time was 0, 6, 25, 29, and 38, respectively. According to Duncan's multiple range test ($P = 0.05$), 0 and 6 were not significantly different from each other, but were significantly different from 25, 29, and 38%. Assuming clubroot symptoms are

the result of II° zoospore infection, cortical infection occurred within 3 yr after II° zoospores were present.

DISCUSSION

By controlling the ψ_m, infection processes could be terminated after specified periods. Dropping ψ_m below −800 mbars prevented root-hair infection, whereas dropping the ψ_m below −150 mbars stopped cortical infection (3). The discontinuous soil-cylinder system allowed plant growth and club formation at a ψ_m that was unfavorable for further cortical infection, allowing interpretation of cortical infection processes started weeks earlier in short-lived membrane systems.

A direct progression of developmental stages in the life cycle of *P. brassicae* was observed. Within 1 day root hairs were infected by I° zoospores arising from resting spores, confirming the report of Williams et al (6). One day later mature sporangia appeared, but no II° zoospores or cortical infections were observed. After 3 days, mature sporangia, II° zoospores, and cortical infection all occurred. This, plus the fact that cortical infection occurred within 3 hr after II° zoospores were present, is evidence that I° zoospores do not lead to club formation. The consistency of results obtained by the different methods supports the validity of these conclusions.

That cortical infection requires a higher ψ_m than root-hair infection (3) most likely reflects the need for more and larger water-filled soil pores required by fused, and therefore larger and perhaps less active, zoospores. If zoospore fusion is required for cortical infection, it appears from this study that only II° and not I° zoospores are involved. The question of whether sufficient numbers of I° zoospores were present to obtain a significant number of fusions and consequent infection is open to further experimentation. In most experiments, between 40–80% of the root hairs were infected and many root hairs had more than one sporangiosorus, indicating that multiple infection had occurred (4). It was estimated that about 60% of the sporangia matured within 24 hr, suggesting that 60% of the I° zoospores had been active and therefore available for fusions at about the same time. Moreover, increasing the inoculum level of resting spores 100- and 10,000-fold should have increased the probability of I° zoospore fusions, but the results were not altered (*unpublished*). It appears, therefore, that whether or not I° zoospores fused, they were not directly involved in cortical infection.

The postulated requirement for II° zoospore fusion in *P. brassicae* is consistent with the reported necessity for fusion in *Polymyxa betae* Keskin, a close relative of *P. brassicae*, and with much larger and more easily observed zoospores (5). Our work provides additional evidence that the infection cycle proposed by Ingram and Tommerup (4) is correct.

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