# The role of primary and secondary infection in host response to *Plasmodiophora brassicae*

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Short title: Secondary zoospores of *Plasmodiophora brassicae*

**Abstract**

The disease cycle of *Plasmodiophora brassicae* consists of a primary phase in root hairs followed by a secondary phase in the root cortex and adjacent tissues. However, the role of root hair infection in subsequent cortical infection and development of *P. brassicae* is not well understood. To examine the role of the primary and secondary stages separately, inoculation studies with resting spores (source of primary zoospores) and secondary zoospores of a virulent and avirulent pathotype were conducted on canola (*Brassica napus*). The size of secondary zoospores and number of nuclei were also examined. The zoospores were larger (~ 9.6-14.4 μm) than in previous reports and all were uni-nucleate. Inoculation with secondary zoospores alone produced both primary and secondary infection, even with the avirulent pathotype. No symptoms developed from inoculation with avirulent primary zoospores, but tiny, bead-shaped clubs developed from inoculation with avirulent secondary zoospores. Inoculation with virulent secondary zoospores alone resulted in lower disease severity than inoculation with virulent resting spores alone. The results indicate that recognition of infection by the host, and initiation of a response (induction or suppression of resistance) occurs during primary infection, although recognition can also occur during cortical infection and development.

***KEY WORDS*:** clubroot, cortical infection, zoospores, resistance, canola

## Introduction

*Plasmodiophora brassicae* Woronin, an obligate parasite causing clubroot, is an emerging threat to canola (*Brassica napus* L.) production in Canada (21, 23) and across the Northern Great Plains of the United States. The disease cycle of *P. brassicae* consists of two phases, a primary phase that is restricted to root hairs and occasionally epidermal cells, and a secondary phase that involves pathogen infection and proliferation within the root and subsequent symptom development (24). In the primary phase, resting spores in the soil germinate and release primary zoospores that penetrate root hairs to form primary plasmodia. The primary plasmodia form multinucleate zoosporangia that produce uni-nucleate secondary zoospores. This process takes about 5 days at the optimum temperature of 25° C (38). The secondary zoospores are released into the soil solution, infect root cortical cells, and develop into motile secondary plasmodia (10, 27). After a mobile phase within the root, the young plasmodia coalesce into sessile vegetative plasmodia that divide to form long-lived resting spores (10, 24, 27). Cortical infection results in hyperplasia and hypertrophy of the root cells, which produces the characteristic swollen, rounded clubbed root symptoms (24, 27). Clubbed roots are readily visible within 5 to 6 weeks of inoculation at temperatures of 20–25° C (39).

The roles of primary and secondary infection in pathogenesis by *P. brassicae* are not fully understood. Primary and secondary zoospores cannot be differentiated visually; both are ovate with two whiplash flagella of unequal length (12,13, 24). Some authors, and at least one authoritative pathology textbook, have concluded that secondary zoospores fuse to form bi-nucleate zoospores prior to infecting the root cortex (2, 10, 24),

but observation of fusion has not been reported (10). Thus, it is still unclear whether fusion of secondary zoospores is a necessary step prior to cortical infection.

Inoculation with resting spores initially produces only primary (root hair) infection, but inoculation with secondary zoospores results in both primary and secondary infection in susceptible hosts (12, 35). Primary infection occurs in both susceptible and resistant host cultivars (6, 8, 15, 34). Primary infection can also occur in several non-host species (34), but secondary infection and development of small numbers of resting spores has been reported in only a fewspecies that are generally considered non-hosts(33).

It is not known whether primary infection affects the initiation of resistance in host species. However, recent studies indicate that non-host resistance to secondary infection is induced initially during root hair infection. Inoculation of a non-host, perennial ryegrass (*Lolium perenne* L.), with secondary zoospores resulted in secondary infection and production of young plasmodia, but not mature plasmodia or resting spores (12).

The predominant pathotypes of *P. brassicae* in Canada are pathotypes 2, 3, 5 and 6 (based on the differential set of Williams (45) and confirmed using the European Clubroot Differential set (42, 43). Several pathotypes of *P. brassicae* are present on the Canadian prairies, and more than one pathotype can be present in a field (4, 43, 46). However, pathotype 3 is the predominant pathotype found on canola in the region (4). Breakdown of resistance to *P. brassicae* has been documented in numerous Brassica species (9, 32). Loss of genetic resistance is a concern for Canadian canola producers (23, 32), where resistance represents one of the most effective tools to manage clubroot (18).

The current study was conducted to assess the roles of infection by primary and secondary zoospores in the initiation of susceptibility and resistance to *P. brassicae* in canola. Experiments were conducted to compare both compatible (susceptible host) and incompatible (resistant host) reactions of canola plants inoculated with primary and /or secondary zoospores of virulent and avirulent pathotypes of *P. brassicae*. The treatments were selected to permit evaluation of two competing hypotheses of disease reaction to clubroot. One hypothesis was that primary infection suppresses initiation of resistance in a susceptible cultivar and stimulates initiation of resistance in a resistant cultivar. The alternative was that primary infection has no effect on initiation of resistance. In addition, flagellate and encysted secondary zoospores were examined to determine whether they were uni- or bi-nucleate and to confirm their morphology.

## Materials and Methods

### Plant material, inoculum, and inoculation

The canola cv. 'Zephyr' (AAFC, Saskatoon, SK, Canada) was selected for the study because it is resistant to pathotype 6 (P6) of *P. brassicae* but susceptible to P3 (Deora, unpublished). All plants were watered daily with demineralised water adjusted to pH 6.3 using commercial vinegar (5% acetic acid), and fertilized weekly with a nutrient solution composed of 0.025% each of NPK (20:20:20, Plant Products Co. Ltd., Canada).

A field isolate of P3 was obtained from clubbed roots of canola grown in a commercial field near Edmonton, AB, Canada, where P3 is predominant (42, 43). An isolate of P6 (S. Strelkov, personal communication) was obtained from clubbed roots of canola grown at the Muck Crops Research Station of the University of Guelph, Holland Marsh, ON, Canada. Inoculum of each pathotype was increased prior to the study on the

highly susceptible Shanghai pak choy cv. Mei Qing Choy (Stokes Seeds, St. Catharines, ON, Canada) under controlled conditions (40). The clubbed roots were stored at -20° C prior to use.

Inoculum of resting spores was prepared based on the method of Jones et al. (26). Briefly ~3 g of frozen clubs (swollen clubbed roots) were thawed at room temperature, homogenized in 100 ml of water at high speed for 2 min, and the resulting spore suspension was strained through eight layers of cheesecloth. The resting spore concentration was determined using a haemocytometer and adjusted to $3 \times 10^5$ spores $ml^{-1}$. Freshly prepared inoculum was used for each inoculation. Five-day-old seedlings were inoculated by pipetting 5 ml of the spore suspension at the base of each seedling and control plants were mock inoculated with deionized water.

Inoculum of secondary zoospores for an initial trial in cell culture plates was produced using the method of Feng et al. (12) with only slight modification. Seed was planted in 20 cm × 20 cm × 10 cm plastic trays filled with washed, autoclaved coarse sand. Seven-day-old seedlings were inoculated with 500 ml of $1 \times 10^8$ resting spores $ml^{-1}$. To avoid cross-contamination, separate trays were used for each pathotype. After 7 days, about 4000 plants were uprooted and the roots were washed with tap water. The foliage was then cut off near the hypocotyl, and the roots were further rinsed three times by shaking at 150 rpm for 20 min in 200 ml of sterile deionized water in a 500 ml flask. The roots (~1000 per flask) were shaken for 20 h in 50 ml deionized water at 100 rpm to stimulate the release of secondary zoospores. After removing the roots, 10 ml of the zoospores suspension was concentrated by centrifugation at 5000 g for 5 min and adjusted to $1 \times 10^6$ zoospores $ml^{-1}$. Ten samples of this suspension were examined

microscopically to confirm that no resting spores were present in the suspension. The concentration of secondary zoospores in the initial inoculum suspension was estimated using a haemocytometer and adjusted to $3 \times 10^5$ spores $ml^{-1}$. The spore suspension was then used immediately for inoculation.

For the study of symptom development, secondary zoospores were produced on canola seedlings planted in Conetainers (plastic 21 cm × 3.8 cm cones Conetainers, Stuewe and Sons Inc., Corvallis, OR) filled with sand, thinned to five seedlings per cone, and inoculated with 5 ml of $1 \times 10^8$ resting spores $ml^{-1}$ per cone at 5 days after seeding (DAS). The seedlings were washed and then planted directly into treatment cones as a source of secondary zoospores at 7 days after the initial date of inoculation with resting spores (12 DAS) (35). This approach was used because it was not possible to harvest the large number of secondary zoospores required using the initial methodology.

**Primary and secondary infection**

Canola seed was sown into 24-well cell culture plates (Sigma Aldrich Canada Ltd., Oakville, ON, Canada) filled with sand. The plates were watered daily and maintained in a growth room at 20°/25° C (day/night) with 75% relative humidity and a 16-h photoperiod. After 5 days, the plates were thinned to one seedling per cell. Each experimental unit consisted of a single seedling, and the study was arranged in a randomized complete block design (RCBD), with four replicates at each of three sampling dates. There were nine inoculation treatments: 1) resting spores (RS) of P3, 2) RS of P6, 3) RS-P3@10 DAS, 4) RS-P6@10 DAS, 5) secondary zoospores (SZ) of P3, 4) SZ-P6, 5) RS-P3+SZ-P3, 6) RS-P6+SZ-P3, and 9) a control inoculated with sterile

water only. All of the resting spore treatments except those designated as '@10 DAS' were applied to 5-day-old seedlings. Secondary zoospore treatments and the late (@10 DAS) resting spore treatments were applied to 10-day-old seedlings. Each seedling was inoculated by pipetting 0.5 ml of $3 \times 10^5$ spores $ml^{-1}$ at the base of the seedling. At optimum temperature for pathogen development, the primary phase of development is nearly complete and secondary zoospores are released at about 5 days after inoculation (39, 40). Therefore, secondary zoospores were applied 5 days later than the primary zoospores to ensure that, as much as possible, secondary infection in all of the treatments occurred at the same time, and that the seedlings were at a uniform developmental stage when secondary infection occurred. The late inoculation treatments with resting spores were included to provide an inoculated control for treatments at 10 DAS.

Four seedlings per treatment were destructively sampled at 11, 13 and 15 days after seeding (1, 3, and 5 days after application of the secondary zoospores and late application of resting spores). The root of each seedling was separated from the foliage, washed in running water, and placed individually in 2-ml centrifuge tubes containing a fixative solution of 50% ethanol. Five fields of view along a 2-cm-long root segment from each taproot were assessed using a compound microscope with a 10x objective lens (39). In each field of view, the incidence of root hair infection and secondary plasmodia in cortical tissue were counted based on the method of Feng et al. (12).

**Symptom development**

Seeds of canola cv. 'Zephyr' were planted in Conetainers filled with soil-less mix (Sunshine mix # 4, Sun Gro Horticulture Canada Ltd, Spruce Grove, AB). A 5-ml pipette

tip was inserted into the soil-less mix in the centre of the pot. The seedlings were thinned to one per pot after 5 days. The same nine treatments described previously were examined in a RCBD with four replicates and 10 plants per replicate. The main group of resting spore suspension treatments (5 ml, $3 \times 10^5$ spores $ml^{-1}$) were applied to 5-day-old seedlings and secondary zoospore treatments were applied to 10-day-old seedlings, as in the initial trial. However, secondary zoospores were applied by removing the 5 ml pipette tip from each pot and planting five infected seedlings into the cavity created by the pipette tip (36). Non-inoculated plants were used as a control. The seedlings used for inoculation were removed after 5 days.

The plants were harvested 52 days after seeding, which was 42 days (6 wks) after inoculation with secondary zoospores. Plant height above the hypocotyl was measured and the roots were washed and assessed for clubroot incidence and severity, based on visual symptoms of root clubbing. The plants were separated into classes using a standard 0–3 scale, where 0 = no clubbing, 1 < 1/3 of the root with symptoms of clubbing, 2 = 1/3–2/3 clubbed, and 3 > 2/3 clubbed (31). A disease severity index (DSI) was calculated using the following equation (43 ):

$$\text{DSI} = \frac{\sum [(\text{class no.})(\text{no. of plants in each class})]}{(\text{total no. plants per sample})(\text{no. classes} - 1)} \times 100$$

Each root was then prepared, sectioned, and stained, and the intensity of cortical infection was assessed using the method of Sharma et al. (39). A segment (0.5-cm-long) was cut from the top 0–1 cm of each taproot, dehydrated through an ethanol series, and a 4-µm-thick cross-section was cut using a microtome. The sections (one per root, four per

treatment) were placed on a microscope slide and stained in 0.5% methylene blue for 5 min. Development of *P. brassicae* was assessed based on the presence of key developmental stages: i) secondary plasmodia, ii) vegetative plasmodia, and iii) resting spores . The number of cells that contained plasmodia or resting spores in each microscope field (10x objective lens) were counted in five fields of view per section. Cortical infection was assessed using image analysis, by estimating the area (%) occupied by plasmodia and resting spores in digital images of each field of view (10x objective lens) using image analysis software (Assess version 2.0, American Phytopathological Society, St. Paul, MN). A total of 20 fields of view were assessed per treatment in the trial. The trial was repeated.

To observe the location and extent of lignification, another set of 4-µm-thick cross-sections (one per root, four per treatment) was placed on a microscope slide and stained with 1% toluidine blue O (TBO) for 3 min. These roots were examined using a compound microscope following the method of Deora et al. (7). Lignified areas stained blue and pectic substances stained pink-purple (36).

**Infectivity of resting spores from bead-shaped clubs**

A study was conducted to determine if the resting spores produced in bead-shaped clubs were infectious. In those treatments where bead-shaped clubs developed, the tap root and lateral roots were collected, washed, diced into small pieces and used to inoculate 5-day-old seedlings, with pieces from two roots applied to each seedling. Roots of non-inoculated seedlings (negative control) and plants that had been inoculated with resting spores of P6 (positive control) were also assessed. The inoculated plants were harvested 6

wk after transplanting and assessed for clubroot incidence and severity as described previously. The trial was laid out in a RCBD with four replicates and 10 plants per replicate. The trial was repeated.

**Nuclear visualization**

The nuclei of zoospores forming within root hairs were stained and photographed. Seedlings of the susceptible canola cv. 46A76 (Pioneer Hi-Bred, Caledon, ON) were grown at 24/20 °C in Conetainers filled with autoclaved (121° C for 30 min) sand. A 5-ml suspension of $1 \times 10^6$ resting spores $ml^{-1}$ of P6, prepared as described previously, was applied at the base of each 8-day-old seedling; the control received water only. There were three replications with three plants per replicate. Plants were watered every day as required. The roots were harvested at 12 days after inoculation and preserved in ethanol: acetic acid (3:1) solution. The harvested roots were stained with Hoechst 33258 (bisbenzimide H, Sigma-Aldrich Canada Ltd.) at 20 µg/ml and observed under an upright Leica DM 6000B confocal laser scanning microscope (CLSM) (Leica Microsystems, Concord, ON Canada). Only root hairs that contained the zoosporangial stages of primary infection were targeted to observe the nuclei of secondary zoospores. An excitation wavelength of 405 nm and an emission and detection channel of 427–490 nm were used. To produce a fluorescence image, averaging (oversampling) was conducted. For Z-sectioning, 10 to 30 sections were obtained, depending on the depth of the sample. For the overlaid image of fluorescence and differential interference contrast (DIC) presented, the thickness of the sample was 35 µm, for which 85 sections were obtained at a step size of 0.42 µm.

To stain the nuclei of flagellate zoospores, 100 µl of secondary zoospores suspension (produced as described previously) was placed on a lysine-coated glass-bottomed culture dish (14-mm-diam., MatTek Corporation, MA). The zoospores were fixed by adding 100 µl of 2% glutaraldehyde. Zoospores were allowed to settle on the bottom of the dish for 30 min, rinsed three times in phosphate buffer (pH 6.8) at 2 min intervals, stained for 1 min with DAPI (4'-6-diamidino-2-phenylindole, 1 µg/ml water, Sigma-Aldrich Canada Ltd.), rinsed with buffer, and mounted on a glass slide in 50% glycerol with 0.1% p-phenylendiamine. The number of nuclei in each zoospore was assessed using epifluorescence microscopy with a blue filter (wide band UV); excitation band pass 360–385 nm (dichromatic beam splitter DM400) and emission barrier filter 420 nm (Olympus BX60F5 microscope, Olympus Optical Co. Ltd., Japan).

For nuclear staining of encysted zoospores, 5 ml of the secondary zoospore suspension was concentrated by centrifugation at 5000*g* for 5 min, and then assessed as described above. At least 25 flagellate or encysted zoospores were observed in each assessment and the study was repeated.

The size of secondary zoospores produced and held in various solutions was assessed. After harvesting the roots for secondary zoospore production as described above, roots were incubated in a shaker in either deionized water, phosphate buffer ($Na_2HPO_4$ and $NaH_2PO_4$; Sigma-Aldrich, St. Louis, MO) or sodium chloride physiological solution (Sigma). The pH of the phosphate buffer and physiological saline was adjusted to 7.0 with NaOH (Sigma). After 20 h of incubation, a suspension of swimming or encysted zoospores (obtained by vortexing for 1 min) from each solution was fixed with 1% glutaraldehyde. The length and width of 30 fixed zoospores from each

solution were measured on photos taken using a stereo binocular microscope (Nikon Eclipse 5.1, Nikon Corporation, Japan).

**Statistical analysis**

There were two repetitions of each experiment investigating infection and symptom development. A general linear model analysis of variance was conducted using Proc GLM of SAS version 9.1 (SAS Institute, Inc., 2010, Cary, NC). Prior to analysis, root hair infection (RHI, %), clubroot incidence (%), clubroot severity (disease severity index, DSI), and area of cortical infection (%) data were arcsine transformed to improve the normality and homogeneity of variance, but non-transformed means are presented for uniformity of presentation. There was no repetition effect or repetition × treatment interaction for any response variable, so the repetitions of each trial were pooled for subsequent analysis. Means separation was conducted using Tukey's test at $\alpha \leq 0.05$. The correlation between cortical colonization and disease severity index was examined using Pearson's correlation coefficient in Proc Corr of SAS.

In the infection and symptom development trials, a few specific comparisons were particularly important for hypothesis testing. Comparison of RS-P6 with SZ-P6 provided a measure of the role of primary infection in initiation of resistance to an avirulent pathotype. Similarly, comparison of RS-P3 with SZ-P3 provided a measure of the role of primary infection in suppression of resistance to a virulent pathotype. Comparison of RS-P6+SZ-P3 vs. RS-P3+SZ-P3 provided a measure of the role of early initiation of an incompatible host reaction (P6) or a compatible reaction (P3) prior to secondary infection and subsequent development of the virulent pathotype (SZ-P3). Similarly, comparison of

RS-P6+SZ-P3 with SZ-P3 provides a measure of the impact of early initiation of resistance on subsequent development of a virulent pathotype, and comparison of RS-P3+SZ-P3 with SZ-P3 provides a measure of the impact of early suppression of resistance on subsequent development of a virulent pathotype.

## Results

### Primary infection

Each of the inoculation treatments resulted in root hair infection (RHI, Table 1). At 13 and 15 DAS (3 and 5 days after inoculation), RHI was higher following inoculation with SZ-P3 compared to SZ-P6. Substantial levels of infection were first observed at 1 day after inoculation in plants inoculated with RS-P3+SZ-P3 (63%), RS-P3 (46%) and RS-P6+SZ-P3 (35%). At 15 DAS (5 days after inoculation), RHI was relatively high for all of the treatments except RS-P6@10 DAS (26%), although differences among treatments were still evident. RHI was higher for RS-P3 than RS-P6 and higher for SZ-P3 than SZ-P6. RHI with RS-P3+SZ-P3 was higher than RS-P3 alone.. However, RHI was higher with RS-P3+SZ-P3 than RS-P6+SZ-P3.

The incidence of RHI increased over time. There was a small inoculation treatment x sampling date interaction ($F=148.5$, $P = 0.0001$, as compared to $F= 5308$ for sampling date and 2249 for treatment), but inoculation treatment generally continued to exhibit a similar pattern of response at the later assessment dates to that observed at 11 DAS.

**Secondary infection**

The most important differences in this study were observed in infection of the root cortex (Fig. 1) and subsequent symptom expression (Table 2). Substantial numbers of plasmodia in the root cortex were observed at 11 DAS in plants inoculated with RS-P3+SZ-P3 (15 secondary plasmodia) and RS-P3 (10 secondary plasmodia). Plasmodia were first observed at 13 DAS in plants inoculated with RS-P3@10 DAS and SZ-P6, and at 15 DAS in plants inoculated with RS-P6@10 DAS. Plasmodia produced from primary and secondary zoospores were similar in morphology (Fig. 2 A, B).

By 15 DAS, the differences among treatments were even more distinct than at the earlier assessment dates (Fig. 1). As expected, inoculation with RS-P3 resulted in many more secondary plasmodia per field of view (mean of 24 plasmodia per field) than RS-P6 (12 plasmodia). Inoculation with secondary zoospores alone resulted in fewer plasmodia than resting spores of the same pathotype; 12 plasmodia for RS-P6 vs. 9.5 plasmodia for SZ-P6, and 24 plasmodia for RS-P3 vs. 16 plasmodia for SZ-P3. RS-P3+SZ-P3 resulted in more plasmodia (30 plasmodia) than either SZ-P3 alone (15 plasmodia) or RS-P3 alone (24 plasmodia). RS-P6+SZ-P3 resulted in fewer plasmodia than RS-P3+SZ-P3 (15 vs. 30). There were no differences in the number of plasmodia when SZ-P3 was applied alone or in combination with RS-P6.

**Symptom development**

The pattern of symptom development and the extent and development of cortical infection were evaluated at 52 DAS. The area of cortical infection, clubroot severity, and the number of cells containing resting spores all showed the same pattern of response to

inoculation treatment (Table 2). The area of cortical colonization and the disease severity index were highly correlated ($r = 0.92$; $P < 0.0001$). These trends were similar to that of early (15 DAS) cortical infection above, except that no symptoms developed on plants inoculated with RS-P6, even though some plasmodia had developed by 15 DAS and young plasmodia were still visible at 47 DAS (22 infected cells per field of view).

In both the compatible and incompatible interactions, inoculation with resting spores elicited a different pattern of response than inoculation with secondary zoospores (Table 2). Inoculation with RS-P3 resulted in the highest values for both area of cortical infection (33%) and number of cells with resting spores (32 cells), and 100% clubroot incidence and severity. SZ-P3 produced less cortical infection (12%) and lower incidence and severity (78% and 67% DSI) than RS-P3. The opposite pattern was observed in the incompatible interaction; inoculation with RS-P6 produced 0% clubroot incidence (no symptoms), but inoculation with SZ-P6 produced low levels of cortical infection (4%) and development of resting spores (2 cells). Clubroot symptoms developed following inoculation with SZ-P6, with a low incidence and severity consisting solely of small bead-shaped clubs.

RS-P3+SZ-P3 produced 100% incidence and severity and the highest cortical infection and number of cells with resting spore, the same as RS-P3 alone (Table 2). However, RS-P6+SZ-P3 resulted in reduced levels of pathogen development in the root cortex: incidence and severity were lower (85% and 86% DSI), as was the area of cortical infection (18%) and number of cells with resting spores (26 cells). The only unusual result was the comparison of SZ-P3 with RS-P6+SZ-P3. Inoculation with RS-P6+SZ-P3

resulted in higher area of cortical infection, more resting spores, and higher clubroot severity than SZ-P3 alone.

No clubs developed on plants inoculated with RS-P6 or on the non-inoculated control (Table 2). Typical large clubs developed on all of the other treatments. The only exception was SZ-P6, where tiny, bead-shaped clubs developed in strings or clusters along the root (Fig. 2C, D, E). Resting spores were present in the infected cortical cells of the bead-shaped clubs (Fig. 3F). About 20 plants were assessed after an additional 2 wk of growth (8 wk after inoculation), and the size and shape of the clubs had not changed.

Plant height and shoot weight were generally highest, and root mass and clubroot incidence and severity were lowest, in plants with no clubbing (inoculated with RS-P6 or controls) or with bead-shaped clubs (SZ-P6), intermediate with RS-P6+SZ-P3 and SZ-P3, and lowest / highest in plants with large clubs (RS-P3 and RS-P3+SZ-P3) (Table 2). Similarly, the root biomass of plants inoculated with RS-P6 (1.1 g) and SZ-P6 (1.4 g) were similar, but only SZ-P6 produced clubroot symptoms, although the bead-shaped clubs were considerably smaller in size and weight than typical clubs (Table 2).

Proliferation of *P. brassicae* resulted in severe distortion of root growth and development (Fig. 3). In cross-sections of the roots of control plants, the periderm, cortex and stele region were organised and continuous (Fig. 3A, B). In plants inoculated with RS-P6, young plasmodia were observed in the cortical cells and stele, but the pathogen did not develop further (Fig. 3C, D). As a result, plants inoculated with RS-P6 did not differ morphologically from the controls. Typical clubs developed from inoculation with RS-P3, RS-P3+SZ-P3, SZ-P3, and RS-P6+SZ-P3, but the clubs from inoculation with SZ-P3 and RS-P6+SZ-P3 were smaller than normal. In all of the typical clubs, cortical

cells of the infected roots became enlarged and intermingled with the secondary phloem, and the distinct organization of tissues into cortex, endodermis and stele was lost (Fig. 3G, H). The area occupied by cells with walls that stained for lignin in the stele decreased rapidly during pathogen colonization (Fig. 3G, H).

In the bead-shaped clubs, cortical cells were highly plasmolysed and the stele was continuous (Fig. 3E, F). The pathogen did not penetrate into the stele. As a result there was limited cell proliferation in these tissues (Fig. 3F). Plants inoculated with resting spores from the bead-shaped clubs became heavily infected and produced large clubs.

**Nuclear visualization**

Zoosporangia were present in most of the infected root hairs at 12 days after inoculation. In mature zoosporangia, the plasmodial cytoplasm was cleaved to produce individual zoosporangia (Fig. 4A). CLSM observation showed that these zoosporangia were multi-nucleate and each nuclei reflected production of uni-nucleate incipient secondary zoospores within the root hairs. Depending on the size, 1–6 nuclei were present in a zoosporangium ($n$ = 20) and the nuclear size varied from 1.0 to 1.5 µm ($n$ = 50) (Fig. 4A).

The flagellate secondary zoospores collected in deionized water had a mean length of 14.4 µm ± 0.19 and width 9.6 µm ± 0.32 (n = 55), and all had two anterior whiplash flagella (Fig. 4B). The mean diameter of encysted zoospores (Fig. 4D) obtained by centrifugation was 21.7 µm ± 0.37($n$ = 50). All of the flagellate and encysted zoospores were uni-nucleate (Fig. 4C, 4D). Secondary zoospores that were released into phosphate buffer or physiological saline were slightly smaller than those produced in

deionized water. The length and width of flagellate zoospores in phosphate buffer were 12.5 µm ± 0.23 x 8.7 µm ± 0.25 and in physiological saline were 11.8 µm ± 0.29 x 8.3 ± µm 0.30. The diameters of encysted zoospores in phosphate buffer and physiological saline were 17.8 µm ± 0.28 and 17.3 µm ± 0.44, respectively.

**Discussion**

This is the first study to compare the roles of primary and secondary infection in both compatible and incompatible reactions between *P. brassicae* and a host. We provide evidence that root hair infection by *P. brassicae* plays an important role in host recognition of the pathogen and influences the development of the pathogen at the cortical infection stage. Canola cv. Zephyr was chosen because it is susceptible to pathotype 3 (compatible reaction) and resistant to pathotype 6 (incompatible reaction); these reactions were consistent across the trials. The severity of clubroot symptoms (DSI) exhibited the same pattern of response as the extent of secondary infection of the root cortex and the number of cells with resting spores, so these are the assessments that the discussion will focus on.

Inoculation with RS-P6 (resting spores of pathotype P6, avirulent on cv. Zephyr) resulted in no symptoms and very limited development of the pathogen, as expected in an incompatible interaction. Inoculation with RS-P3 (resting spores of P3, virulent on cv. Zephyr) resulted in the greatest symptom development and highest number of cortical cells containing resting spores. This was as expected in a compatible reaction (6, 13, 17). Inoculation with secondary zoospores of P6 alone (SZ-P6) resulted in much more cortical

infection than with RS-P6, while inoculation with SZ-P3 alone resulted in less cortical infection than RS-P3. Comparison of the effect of inoculation with resting spores vs. secondary zoospores on cortical infection and DSI provided a measure of the role of primary infection in initiation of resistance or susceptibility. These results demonstrate that primary infection plays a role in subsequent cortical infection in both compatible and incompatible reactions. Cortical infection was almost completely suppressed after primary infection with an avirulent pathotype (RS-P6), but the root cortex was infected and colonized to a substantial extent with secondary zoospores of an avirulent pathotype (SZ-P6). This indicates that primary infection with P6 induced a resistance response that was most strongly expressed in the root cortex, although there was also a small effect on pathogen development in root hairs. Conversely, cortical infection following primary infection with a virulent pathotype (RS-P3) was more extensive than with secondary zoospores of a virulent pathotype (SZ-P3) alone, which indicates that primary infection induced susceptibility or suppressed resistance in the root cortex.

Comparison of RS-P6+SZ-P3 vs. RS-P3+SZP3 or SZ-P3 provided a measure of the impact of early initiation of a resistance reaction on subsequent development of a virulent pathotype. Inoculation with RS-P6+SZ-P3 resulted in less infection and lower severity than RS-P3+SZP3 or SZ-P3. This response provides additional support for the hypothesis that primary infection has a role in inducing resistance in an incompatible reaction. In fact, it provides evidence that primary infection is an important step in recognition of the avirulent pathogen. Similarly, comparison of RS-P3 vs. RS-P3+SZ-P3 or SZ-P3 alone provided a measure of the impact of early suppression of resistance on subsequent development of a virulent pathotype in the compatible reaction. Inoculation

with RS-P3 produced more cortical infection and higher severity than SZ-P3 alone, but the same level of symptom expression and cortical infection as RS-P3+SZ-P3. This indicates that primary infection results in a slight but potentially important suppression of host defences in a susceptible host.

Inoculation with SZ-P6 resulted in infection of the root cortex and pathogen development sufficient to produce viable resting spores. However, symptom development was restricted to small, bead-like clubs. This indicates that if recognition of the avirulent pathotype does not occur at the primary infection stage, initiation of a resistance response was delayed but not completely suppressed.

Inoculation with RS-P3 and RS-P3+SZ-P3 resulted in the maximum possible level of clubroot severity (100 DSI) and the greatest area of cortical infection, which might represent the maximum that can occur in this host-pathogen interaction. The initial inoculation with 5 ml of $10^5$ $ml^{-1}$ of resting spores may have been sufficient to produce 100 DSI. Indeed, there are other reports of 100% clubroot severity with similar (5 ml of 1 x $10^6$ $ml^{-1}$) concentrations of inoculum (7, 8. Repeating this comparison with a lower inoculum concentration might help to elucidate the role of primary infection in a compatible interaction.

The only result that did not support a role for primary infection in the initiation of a resistance reaction in the host was the comparison of RS-P6+SZ-P3 vs. SZ-P3. If primary infection by an avirulent pathotype stimulates the early initiation of host resistance, inoculation with RS-P6 before adding SZ-P3 would be expected to suppress infection and symptom development compared to secondary zoospores alone. However, the reverse was observed. There was slightly more cortical infection with RS-P6+SZ-P3

than with SZ-P3 alone (18% vs. 12%), more cells with resting spores (15 vs. 12 cells) and higher clubroot severity (84 vs. 67 DSI). This may indicate that primary infection has a larger role in suppressing host defences than in stimulating resistance. However, additional work in this area is required.

Most physiological and molecular studies on clubroot have focused only on the compatible relationship, and indicate that fewer physiological changes occur in the host during the primary phase of the pathogen's lifecycle compared with the secondary stage (3, 41). However, none of the effectors that mediate compatible and incompatible relationships with *P. brassicae* have been identified or characterized. During infection in many host-pathogen systems, the pathogen secretes effectors that interact with the host and play an important role in virulence and/or the stimulation of a resistance (incompatible) response (16, 20, 25, 28). To establish a successful infection, *P. brassicae* must either suppress host resistance or fail to trigger host resistance. In either case, the response is likely triggered by effectors. In nonhosts, *P. brassicae* triggered a resistance response (33), but root hair infection was critical for induction of resistance to secondary infection and subsequent club formation (12).

In a recent study of gene expression in primary and secondary zoospores of *P. brassicae*, many more genes were up-regulated in primary zoospores than in secondary zoospores (14). The authors suggested that the genes that were up-regulated in secondary zoospores likely contribute to infection of cortical tissue. The results of the present study support their hypothesis that there are specific mechanisms required for secondary infection, in that secondary zoospores can infect the root cortex in the absence of primary

infection, but primary infection has an important role in the recognition of a compatible or incompatible pathotype.

The results of the current study are also consistent with the results of a study conducted to identify changes in host gene expression in *Arabidopsis thaliana* (L) Heynh inoculated with the compatible *P. brassicae* ecotype Co-1 (1). At 4 days after inoculation (which corresponds to primary infection), expression of several genes known to be involved in pathogen recognition and signal transduction was induced. Also, many more host genes were down-regulated than were up-regulated. Some of the down-regulated genes were involved in lignin and salicylic acid biosynthesis, the oxidative burst pathway, and several other defence-related genes. In general, these results support the hypothesis that primary infection suppresses host resistance. They are also consistent with a recent study that identified that a compatible *P. brassicae*/canola interaction is characterized by a lack of the reactive oxygen species and a reduction in lignin relative to an incompatible interaction (6).

Infection and development of *P. brassicae* in the root cortex of susceptible and resistant cultivars has been studied in detail (6, 7, 11, 17, 19, 30). Clubroot resistance was expressed most clearly and consistently in the root cortex, where pathogen development occurred quickly in susceptible cultivars. Very little or no pathogen development was observed in highly resistant radish (30) and canola cultivars (6). In the current study, plasmodia were observed in the root cortex of plants inoculated with resting spores of the avirulent pathotype, but these did not develop to produce resting spores and no clubs developed on the roots. This is consistent with recent studies showing that resistance can

be expressed after some initial colonization of the root cortex in canola (7) and cabbage (17).

The present study provides some insights into the timing of the interaction between effectors from *P. brassicae* and the host. Plants inoculated with resting spores of the avirulent pathotype (P6) did not develop clubs, while direct inoculation with the secondary zoospores of P6 resulted in infection and development of small bead-shaped clubs. This indicates that the initial recognition of the pathogen normally occurs during primary infection, so that the host is able to respond with effective resistance mechanisms when challenged by secondary zoospores. Direct inoculation with secondary zoospores either bypassed this recognition stage or did not provide enough time for the resistance reaction to develop fully. However, the results of this study also showed that recognition of the pathogen can occur during cortical infection and subsequent development of the pathogen. Inoculation with secondary zoospores of the incompatible pathotype (P6) resulted in lower levels of pathogen development than inoculation with secondary zoospores of the compatible pathotype (P3), which in turn was lower than from inoculation with resting spores of P3.

The small, bead-shaped clubs produced by secondary zoospores of P6 in this study appear to be similar to the spheroid galls reported in resistant reactions by Williams (45) and others (29). There is some disagreement in the literature as to whether this is a true resistance response. Rennie et al. (38) point out that several researchers considered these an indication of host resistance because the spheroid clubs (galls) represent a restriction of the pathogen within the host. However, the authors suggest that the formation of spheroid clubs should not be interpreted as complete resistance, since small

numbers of resting spores can develop in these clubs. The results of the current study support the conclusion that this reaction does not represent complete resistance. Instead, small clubs are produced when pathogen development is limited after some development has already occurred in the root cortex. This in turn indicates that some component(s) of host resistance was/were bypassed or delayed. Similarly, bead-shaped clubs were observed on canola inoculated with secondary zoospores produced on a non-host (12). This further supports the hypothesis that direct infection by secondary zoospores bypasses recognition by the host during primary infection. However, clubbing symptoms do not develop fully, likely because initiation of resistance is delayed or effectors at the primary infection stage are required to allow a fully compatible interaction.

Differences in the incidence and development of primary infection of resistant and susceptible host cultivars have been studied in detail (6, 11,). The results of the current study support the observation that both virulent and avirulent pathotypes can infect root hairs, but that the incidence of primary infection is higher in plants inoculated with virulent pathotypes than with the avirulent pathotypes (6, 22). The present study also confirms previous reports that secondary zoospores of *P. brassicae* can initiate both primary and secondary infection (12, 13, 35).

Secondary zoospores were examined for the presence of bi-nucleate zoospores because there were reports that bi-nucleate zoospores of *P. brassicae* result from fusion of two zoospores (5, 44) and that cortical infection occurs after the secondary zoospores fuse in pairs (10, 24). In the current study, all of the secondary zoospores were uni-nucleate, which indicates that the majority of secondary zoospores do not fuse. Further study into this phase of the life cycle is warranted. If fusion of secondary zoospores was

a prerequisite to cortical infection, this would greatly reduce the effective inoculum concentration. The secondary zoospores in this study were morphologically identical to primary zoospores (oval shaped, with one nucleus and two flagella of uneven length), as has been reported in previous studies (12, 13, 27).

The average size of secondary zoospores released in deionized water after mechanical shaking of the roots was 9.6 µm x 14.4 µm, while those released into phosphate buffer were slightly smaller. Secondary zoospores observed within root hairs were reported to be much smaller, 2.5 µm x 3.5 µm (24) or 1.5 x 0.5-0.7 µm (5). Differences in the size of secondary zoospores were also observed when they were switched from 10% glucose solution to water (Feng, unpublished). Since the zoospores in the present study were released in deionized water, it is highly likely that they would take up more water and swell to a larger size than those in a buffer or an ionized solution. Secondary zoospores within a root hair are likely smaller than released zoospores as a result of higher osmotic or physical pressure in the root hairs. This is the first report of the size of free living secondary zoospores.

The methods used to produce and collect secondary zoospores were those of Feng et al. (12). This method has several advantages over using infected plants as a source of secondary zoospores, since the inoculum concentration can be quantified and adjusted as required. However, it proved to be difficult to consistently obtain large quantities of secondary zoospores, so infected seedlings were used as a source of secondary zoospores (35) for some of the studies. The results using this approach were consistent between repetitions of the study and exhibit a similar pattern of response to studies using collected

secondary zoospores, so we conclude that this is a suitable inoculation approach for use in future studies.

In summary, this is the first study to demonstrate that the primary infection phase of *P. brassicae* plays an important role in host recognition of the pathogen. The results support the hypothesis that primary infection suppresses the initiation of resistance in a susceptible cultivar and also stimulates the resistance reaction in a resistant cultivar. It appears likely that pathogen effectors are recognized at the primary infection stage and that this early interaction between host and pathogen contributes to the resistant or susceptible reaction in the host at the secondary infection stage. Induction of susceptibility was more effective than initiation of resistance in the two pathotype/host interactions examined. Recognition of the pathogen as compatible or incompatible also occurred in the root cortex, but the resistance reaction developed more quickly and was expressed more strongly in response to primary infection. Our results were consistent with independent studies of gene expression in the pathogen and host in a compatible interaction. However, additional study at the molecular level is needed to further elucidate the role of primary and secondary zoospores in pathogenesis.

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**Table 1.** Incidence of primary infection (root hair infection, RHI) on canola cv. Zephyr resulting from inoculation with resting spores (RS) and secondary zoospores (SZ) of pathotypes 3 (P3) and 6 (P6) of *Plasmodiophora brassicae,* assessed at 11, 13, and 15 days after seeding (DAS) (1, 3 and 5 days after inoculation with secondary zoospores).

| Spore type & pathotype | Root hair infection (%) | | |
|---|---|---|---|
| | 11 DAS | 13 DAS | 15 DAS |
| RS-P6 (10 DAS)[1] | 2 a[2] | 15 a | 26 a |
| RS-P3 (10 DAS) | 3 a | 26 b | 48 b |
| SZ-P6 | 4 a | 32 c | 55 c |
| SZ-P3 | 10 a | 47 e | 74 e |
| RS-P6 | 22 b | 41 d | 50 b |
| RS-P6+SZ-P3 | 35 c | 55 f | 60 d |
| RS-P3 | 46 d | 64 g | 72 e |
| RS-P3+SZ-P3 | 63 e | 73 h | 80 f |
| Standard error | 0.44 | 1 | 0.7 |

[1]The (10 DAS) treatments were inoculated with resting spores at the same time as the secondary zoospore treatments were applied. All other resting spore treatments were applied 5 DAS

[2]Values are the means of eight replications, with one plant per rep. Means within a column followed by the same letter do not differ at $P < 0.05$ based on Tukey's test.

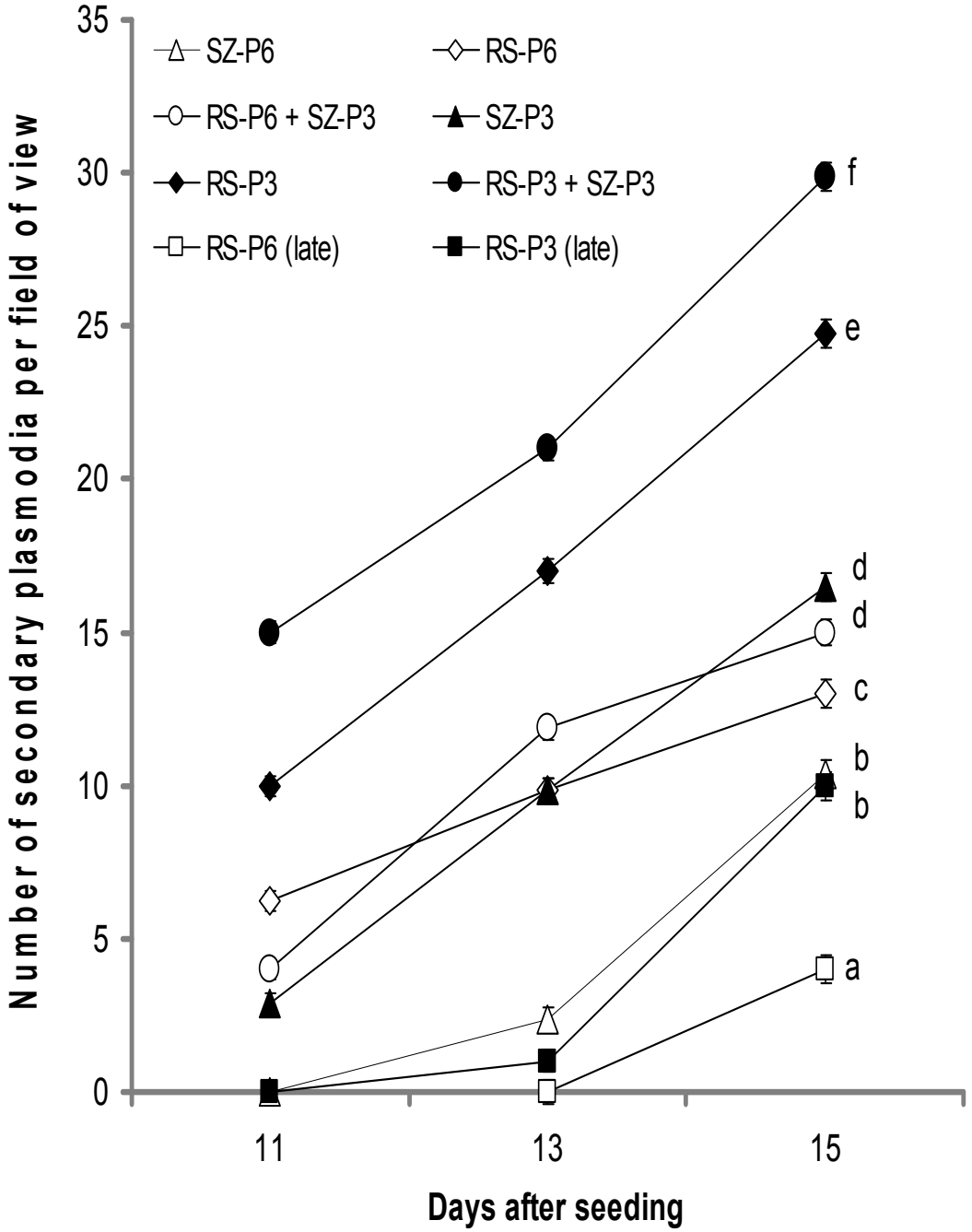


**Fig. 1.** Secondary infection (number of plasmodia per field of view) of canola 'Zephyr'at 1, 3, and 5 days after inoculation with resting sores or secondary zoospores'. The regression slopes among the treatments were similar therefore are not presented. Data points are the means of observed values of eight replications (one plant per rep). Means at 5 days after inoculation with the secondary zoospores followed by the same letter do not differ based on Tukey's test at $P \leq 0.05$. Capped lines = standard error.

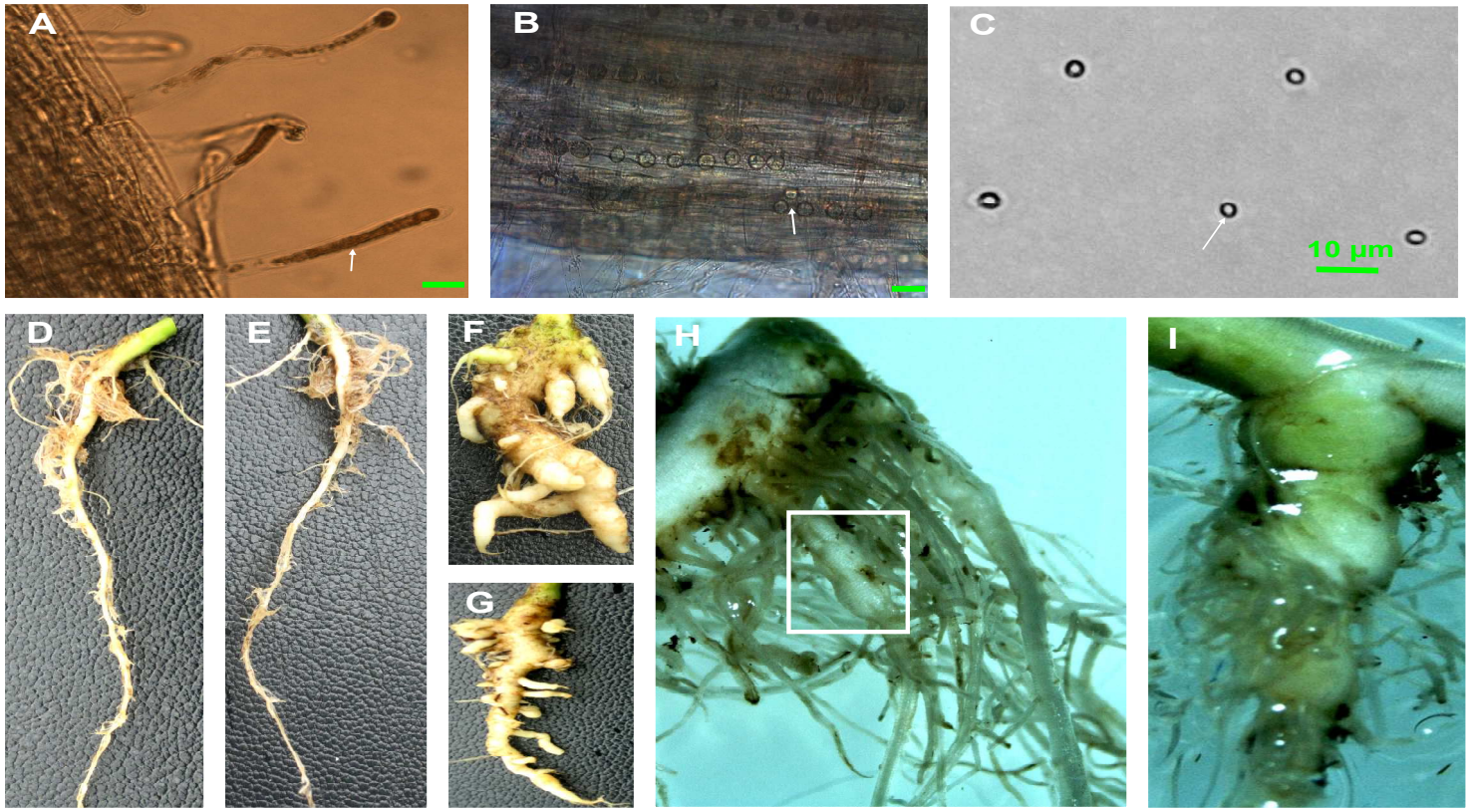


**Fig. 2.** (**A**) Infected root hair (arrow) and (**B**) young secondary plasmodia (arrow) on canola root inoculated with secondary zoospores of pathotype 6 (avirulent on cv. Zephyr) of *Plasmodiophora brassicae*. (**C**, **D, E**) Bead-shaped clubs from inoculation with secondary zoospores of pathotype 6.

**Table 2.** Clubroot severity on harvested roots, and area of cortical infection (%), number of infected cells with young plasmodia, mature plasmodia, or resting spores in five fields of view in sections of canola roots, and mean plant height (cm), shoot weight (g) and root weight (g) of canola cv. 'Zephyr', assessed at 52 days after seeding, which was 47 days after inoculation with resting spores (RS) and 42 days after inoculation with secondary zoospores (SZ) of pathotypes 3 (P3) and 6 (P6) of *Plasmodiophora brassicae*.

| | | | | Number of infected cells | | | Plant growth parameters | | |
|---|---|---|---|---|---|---|---|---|---|
| Spore type & pathotype | Incidence (%) | Severity (%) | Area of cortical infection (%) | Young plasmodia | Mature plasmodia | Resting spores | Plant height (cm) | Shoot wt. (g) | Root wt. (g) |
| RS-P6 | 0 a[1] | 0 a | 0.1 a | 22 d | 0 a | 0 a | 49 c | 4.0 c | 1.1 a |
| SZ-P6 | 67 b | 31 b | 4 b | 13 c | 6 b | 2 b | 54 c | 4.0 c | 1.4 a |
| SZ-P3 | 78 c | 67 c | 12 c | 6 b | 12 d | 12 c | 28 b | 3.0 b | 2.2 b |
| RS-P6+ SZ-P3 | 85 c | 84 d | 18 d | 1 a | 15 e | 26 d | 28 b | 2.6 b | 2.2 b |
| RS-P3 | 100 d | 100e | 33 e | 1 a | 10 c | 32 e | 15 a | 1.3 a | 3.6 c |
| RS-P3+ SZ-P3 | 100 d | 100 e | 34 e | 1 a | 9 c | 32 e | 10 a | 1.0 a | 4.2 c |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 50 c | 4.6 c | 1.1 a |
| Standard error | 0.97 | 1.8 | 0.40 | 0.44 | 0.48 | 0.60 | 1.1 | 0.68 | 0.97 |

[1]Values are the means of eight replications (10 plant per rep for incidence, severity and plant growth parameters, one plant and five fields of view per rep, for data on number of infected cells). Means in the columns followed by the same letter do not differ based on Tukey's test at $P \leq 0$ .05.

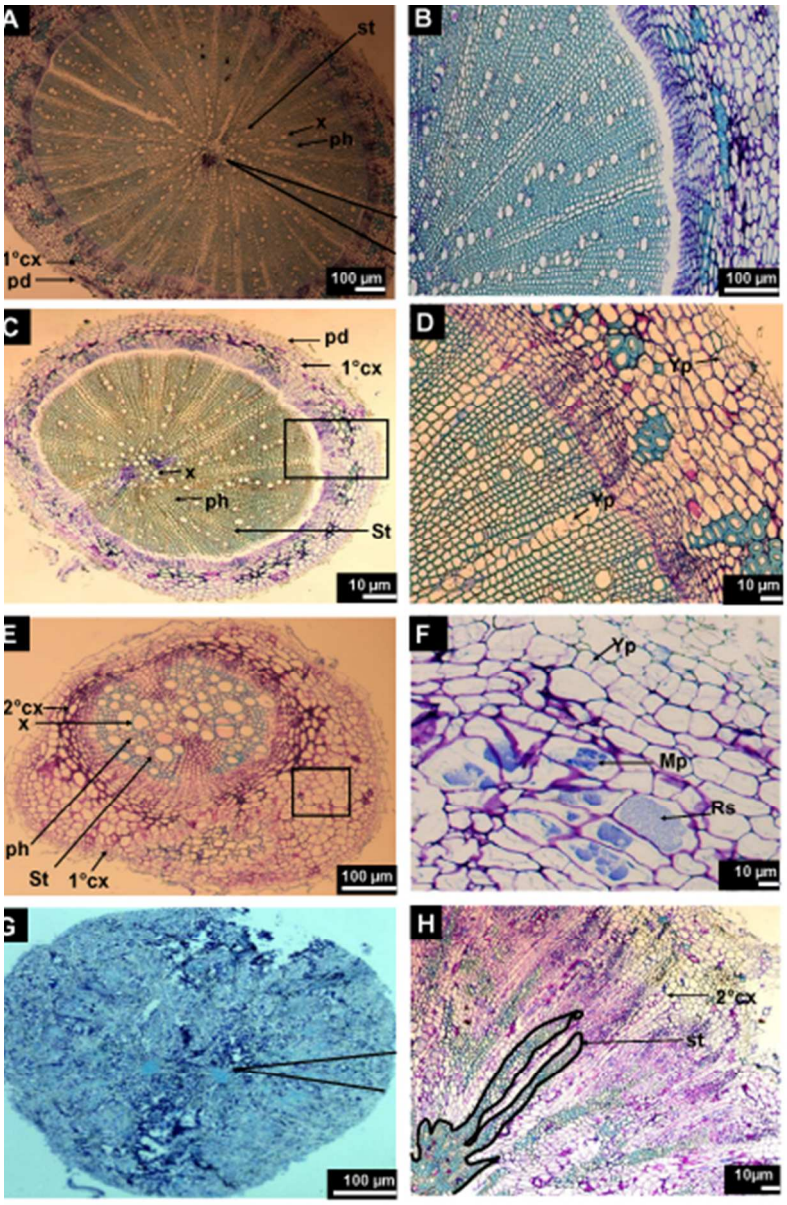


Fig. 3. Cross-section of roots of healthy and P. brassicae-infected canola plants, stained with TBO. (A) Healthy root (control). Lignified xylem cell walls stain dark blue, non-lignified walls are pink. (B) Magnified region from (A) showing intact stele, cortex and periderm. (C) Roots of plants inoculated with resting spores of pathotype 6 (avirulent on cv. Zephyr. (D) Magnified region from (C) showing intact stele, cortex and periderm (young plasmodia are marked with arrow). (E) Bead-shaped clubs produced after inoculation with secondary zoospores of P6. Note the proliferation of cortical tissue outside of the stele, and no proliferation within the stele. (F) Magnified region from (E) showing young plasmodia, mature plasmodia and resting in the cortical cells. (G) Typical clubs. Stele region is disrupted. (H) Magnified region from (G) showing invasion and expansion of stele (marked with black boundaries). Annotations: x, xylem; ph, phloem; 1° cx, primary cortex; 2° cx, secondary cortex; pd, periderm; st, stele.
146x222mm (72 x 72 DPI)

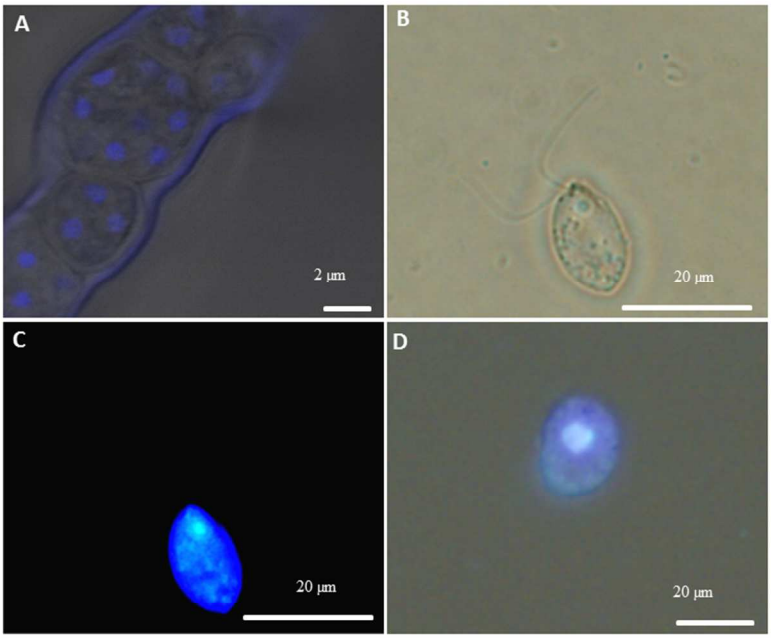


Fig. 4. Developmental stages and nuclear visualization in secondary zoospores (A) CLSM micrograph (overlaid fluorescence and DIC image) of an infected root hair stained with Hoechst 33258 showing the plasmodial mass cleaved into zoosporangia. Each zoosporangium contains a varying number (1 to 6) of nuclei that represent incipient secondary zoospores. (B) to (D) Epifluorescence micrographs. (B) Phase contrast of a bi-flagellated zoospore produced by mechanical shaking of root hairs. (C) Fluoresce image of DAPI staining of a single nucleus in the flagellated zoospore. (D) An overlaid image (fluorescence and phase contrast) of an encysted uni-nucleate zoospore (produced by centrifugation).
254x190mm (96 x 96 DPI)