Germination of surface-disinfected resting spores of *Plasmodiophora brassicae* and their root hair infection in turnip hairy roots

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Germination of surface-disinfected resting spores of *Plasmodiophora brassicae* and its infection of turnip hairy root hairs were studied. Surface-disinfected resting spores showed higher germination than non-disinfected resting spores. Root hair infection was most frequent in the section of root formed 1 d before inoculation. Root hair infection began 4 d after inoculation, increased up to 6 d, and continued to increase more slowly until 10 to 12 d after inoculation. Growth of *P. brassicae* in the root hair of hairy roots was observed serially. Most primary plasmodia differentiated to mature zoosporangia 8–10 d after inoculation. The secondary zoospores were initially released 6 d after inoculation.

Key Words——hairy root; *Plasmodiophora brassicae*; root hair infection; serial observation.

Clubroot is an economically important soil-borne disease of cruciferous crops caused by *Plasmodiophora brassicae* Woronin. The pathogen is an obligate parasite, and its life cycle consists of two phases. The first phase is the development of primary plasmodia within the root hair, and the second is the infection in the living cells of the root cortex and stele leading to abnormal development (Ingram and Tommerup, 1972).

In recent decades, several workers have examined the germination of resting spores of *P. brassicae*. Macfarlane (1970) and Suzuki et al. (1992) found that resting spores derived from matured or rotted galls had higher germinability than those derived from whitish and young galls. Stimulation of germination of resting spores in the presence of host plants or root exudates was also noted by several researchers (Bochow, 1965; Macfarlane, 1970; Suzuki et al., 1992). However, spore germination is not reproducible because experiments cannot be performed axenically.

Several reports have described the penetration and growth process of *P. brassicae* in root hairs of cruciferous crops (Ayers, 1944; Samuel and Garrett, 1945; Katsura et al., 1970; Aist and Williams, 1971; Ingram and Tommerup, 1972). A primary zoospore, which germinates from a resting spore, attaches to a root hair of the host plant. The zoospore then encysts and forms an appresorium, though which the pathogen penetrates the root hair cell wall. A primary plasmodium grows within root hair cells and differentiates into a number of zoosporangia, each bearing 4–16 secondary zoospores. Then secondary zoospores are released though a gap in the root hair cell wall (Ayers, 1944) or into the root hair lumen (Ingram and Tommerup, 1972). How-

ever, with the exception of callus culture (Ingram, 1969; Tommerup and Ingram, 1971), the previous studies were not conducted under axenic conditions. Therefore, the influences of other microorganisms, such as antagonism and induced systemic resistance, can not be avoided. In vitro study is essential to develop a better understanding of plant-parasite interactions.

In recent studies, hairy root cultures have been used to investigate host-parasite interactions (Becaed and Fortin, 1988; Diaz, 1995; Plenchette, 1996). Dual cultures of *P. brassicae* and hairy roots have been studied by several researchers (Muginer, 1987; He, 1991; Graveland et al., 1992). However, surface-disinfestation of resting spores was incomplete, and inoculation was not always done under sterile conditions in these studies. Therefore, consistent results were not obtained in terms of root hair infection and gall formation. Asano et al. (1999) developed a surface-disinfestation method for resting spores of *P. brassicae* and established a technique to induce root hair infection and gall formation in the turnip hairy root.

In the present study, we attempted to make a comparison of germination of surface-disinfected and non-disinfected resting spores of *P. brassicae*. We also observed growth of the pathogen within root hair under axenic conditions.

Materials and Methods

Hairy root culture Two strains of hairy roots of turnip (*Brassica rapa* var. *rapifera* Merz. cv. Wase Okabu), BR.123 and BR.1724, were used in this study. The hairy roots were routinely maintained on Murashige and

Skoog medium (Murashige and Skoog, 1962) in Petri dishes at 25°C in a 16 h light and 8 h dark photoperiod with daytime lighting of approximately 250 μE/m^2/sec.

Clubroot population   A field population of *P. brassicae* was obtained from clubs of clubroot-resistant turnip cv. Strong CR75 grown on a naturally infested field at Aichi, Japan, and maintained on turnip cv. Wase Okabu. The field population was characterized as ECD race coding numbers 16/04/08 and named Toyohashi population. The Toyohashi population has been used in previous studies (Kageyama et al., 1995; Ogawa, 1998), and its resting spores showed similar germination to other populations (Ogawa, 1998). Therefore, we used Toyohashi population.

Surface-disinfestation of resting spores   Surface-disinfestation of resting spores was performed according to Asano et al. (1999). Resting spores were obtained from 8-wk-old galls grown in a greenhouse. The galls were washed thoroughly under running tap water to remove soil particles. The outer layers of the gall tissues were removed and the central parts were surface-sterilized with 70% ethanol for 1 min and 10% sodium hypochlorite (available chlorine 0.5%) for 20 min. The galls were rinsed three times in sterile distilled water, then cut into small pieces. The gall pieces were macerated in a blender in 50 ml of sterile distilled water for 3 min and filtered through eight layers of cheesecloth. The filtrate was centrifuged at 1,700 g for 5 min, and the resulting pellet was washed five times with sterile distilled water. A spore suspension was prepared using sterile distilled water and its concentration was adjusted to 5.0 × 10^7 spores/ml. The spore suspension was centrifuged at 1,700 g for 5 min, and the supernatant was discarded. Resting spores were suspended in freshly prepared 2% chloramine-T solution (w/v) (Wako Pure Chemical Industries Ltd., Osaka, Japan) at room temperature (20–25°C) for 20 min. They were washed twice in sterile water by centrifugation as above. The resulting pellet was suspended in a solution of antibiotics, 1 μg/ml of colistin sulfate (Wako), 1 μg/ml of vancomycin hydrochloride (Wako), and 6 μg/ml of cefotaxime sodium (Hoechst Pharmaceuticals and Chemicals Co. Ltd., Tokyo, Japan), in distilled water and incubated at 25°C in the dark for 1 d. The suspension was washed twice in sterile water by centrifugation. For the non-disinfected control, treatments of resting spores with chloramine-T and antibiotics were omitted.

Resting spore germination   Surface-disinfected resting spores were suspended in 1/10 strength of modified Hoagland's solution (5 mM Ca(NO$_3$)$_2$, 5 mM KNO$_3$, 2 mM MgSO$_4$, 2 mM KH$_2$PO$_4$, pH 5.8) (Macfarlane, 1970) with 0.1 μg/ml of cefotaxime sodium. Ten-ml centrifuge tubes containing 5 ml of resting spore suspension (1.0 × 10^7 spores/ml) were incubated in the dark at 25°C. Resting spore germination was assessed at 2-d intervals using a Nomarski differential interference contrast optic microscope (Olympus Optical Co. Ltd., Tokyo, Japan). About 10 μl of spore suspension was placed on glass slides, and germinated and ungerminated resting spores were directly counted. At least 1200 spores were

randomly counted per specimen.

Inoculation with surface-disinfected resting spores   Turnip hairy roots were inoculated with surface-disinfected resting spores (Fig. 1). Hairy roots were cut into approximately 3-cm lengths and placed on MS medium in 9-cm Petri dishes. The cultures were incubated in the dark at 25°C for 3 or 5 d to allow growth of root hairs. Then 5 ml of disinfected resting spores (5.0 × 10^6 spores /ml in 1/10 strength of Hoagland's solution supplemented with 0.1 μg/ml of cefotaxime sodium) was put over the hairy roots, and the plates were incubated in the dark at 25°C. Root hair infection in terms of infection rate, infection timing, and growth of *P. brassicae* within root hairs was determined in six sections of root formed on succesive days after transfer to MS medium (Fig. 1). Observations were made using an inverted microscope
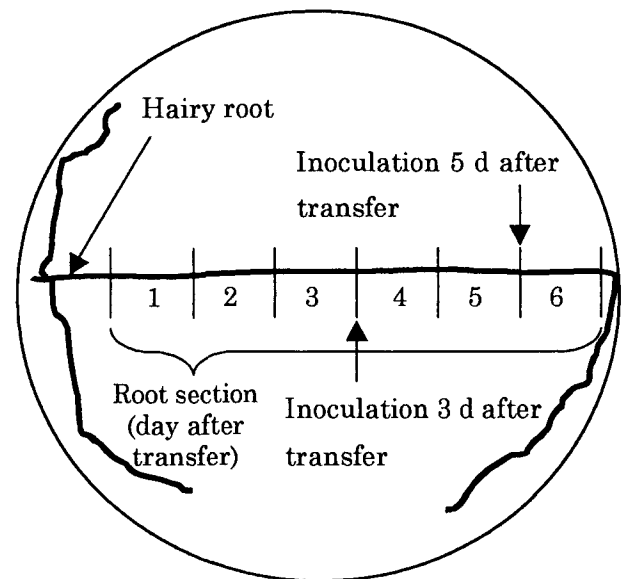

Fig. 1.   Schematic view of inoculation of turnip hairy roots with surface-disinfected resting spores.
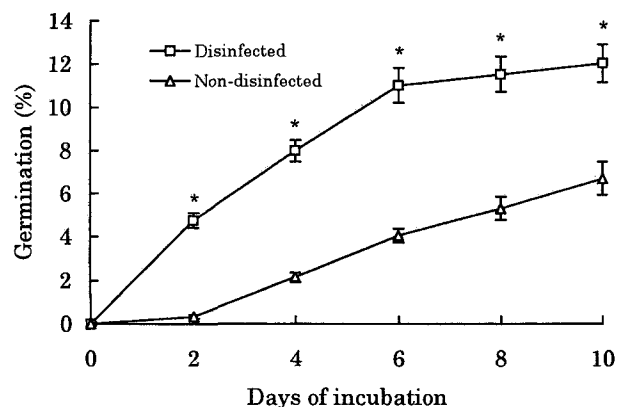

Fig. 2.   Germination of surface-disinfected and non-disinfected resting spores of *Plasmodiophora brassicae*. Vertical bars are standard error and asterisks indicate significant difference ($P < 0.05$) according to t-test.

(Olympus).

Data analysis A completely randomized design was employed in all experiments. The experiments were repeated two or three times with ten replicates per treatment. The means were separated by t-test ($P<0.05$) or Fisher's protected least significant difference test ($P<0.05$).

Results

Germination of resting spores The germination of surface-disinfected resting spores increased steadily for 6 d, then became static, whereas that of non-disinfected resting spores increased slowly until 10 d (Fig. 2). At the end of the experiment, the germination rate of sur- face-disinfected resting spores (12.0%) was significantly higher than that of non-disinfected resting spores (6.7%).

Effect of age of root on root hair infection In roots inoculated 3 d after transfer, the number of root hair infections in both strains was significantly higher in the section formed on the 2nd day after transfer than other sites (Table 1). However, in roots inoculated 5 d after transfer, the root hair infection in both strains was highest in the section formed on the 4th day after transfer. The 14-d-old roots showed highest infection. In other words, root hair infection was frequently observed in the section formed 1 d before inoculation, regardless of the inoculation timing.

Time of root hair infection Table 2 shows the course of

Table 1. Relationship between root hair infection by *Plasmodiophora brassicae* and the age of hairy root 12 d after inoculation.

Root section (day after transfer)	Inoculation 3 d after transfer			Inoculation 5 d after transfer		
	Root age[a]	Number of root hair infections		Root age	Number of root hair infections	
		BR.1724	BR.123		BR.1724	BR.123
1	15	9.65 b[b]	7.60 b	17	6.85 a	2.07 a
2	14	14.19 c	14.36 c	16	7.96 a	1.78 a
3	13	10.08 b	8.20 b	15	13.84 ab	3.41 a
4	12	5.50 a	4.64 ab	14	17.23 b	12.67 b
5	11	4.81 a	2.84 a	13	13.07 ab	4.52 a
6	10	2.62 a	1.33 a	12	13.11 ab	3.41 a

a) Age of root 12 d after inoculation.
b) Means followed by the same letter(s) in a column are not significantly different ($P<0.05$) according to Fisher's protected least significant difference test.

Table 2. Time of infection of root hairs of hairy root by *Plasmodiophora brassicae*.

Root section (day after transfer)	Number of root hair infections											
	Inoculation 3 d after transfer						Inoculation 5 d after transfer					
	Days after inoculation						Days after inoculation					
	2	4	6	8	10	12	2	4	6	8	10	12
BN.1724												
1	0.0	7.1	2.0	1.5	0.7	0.0	0.0	4.5	2.1	0.7	0.3	0.0
2	0.0	9.8	3.2	0.4	0.2	0.0	0.0	7.4	2.5	1.1	0.2	0.0
3	0.0	6.4	2.7	0.2	0.1	0.0	0.0	14.8	3.0	0.6	0.7	0.0
4	0.0	2.1	2.6	0.6	0.5	0.2	0.0	15.9	3.3	0.9	1.3	0.0
5	0.0	0.7	3.6	0.8	0.4	0.0	0.0	12.1	2.5	0.9	1.5	0.0
6	—[a]	0.0	2.1	1.3	0.1	0.2	0.0	3.2	14.1	1.9	0.7	0.0
BN.123												
1	0.0	4.8	1.0	0.1	0.2	0.0	0.0	0.9	1.1	0.1	0.1	0.0
2	0.0	10.1	3.0	0.2	0.1	0.0	0.0	1.1	0.5	0.2	0.1	0.0
3	0.0	5.0	2.3	0.7	0.4	0.0	0.0	1.9	1.5	0.2	0.2	0.0
4	0.0	2.0	2.6	0.5	0.4	0.0	0.0	10.2	2.4	0.9	0.1	0.0
5	0.0	0.0	2.0	0.3	0.8	0.0	0.0	2.2	1.5	0.4	0.3	0.2
6	—	0.0	0.5	0.1	0.6	0.0	0.0	0.6	2.6	0.3	0.4	0.0

a) The root was not yet grown at this time.

root hair infection at 2-d intervals. In both hairy root strains, infection began 4 d after inoculation, increased up to 6 d, and continued to increase more slowly until 10 -12 d after inoculation in almost all sections, regardless of the time of inoculation.

Table 2 also reveals two similar patterns of infection in both strains. One pattern showed most root hair infection 4 d after inoculation. This infection occurred in the sections formed before inoculation (days 1-3 and 1-5 after transfer respectively for roots inoculated 3 d and 5 d after transfer). The other pattern showed the peak of root hair infection 6 d after inoculation. This infection
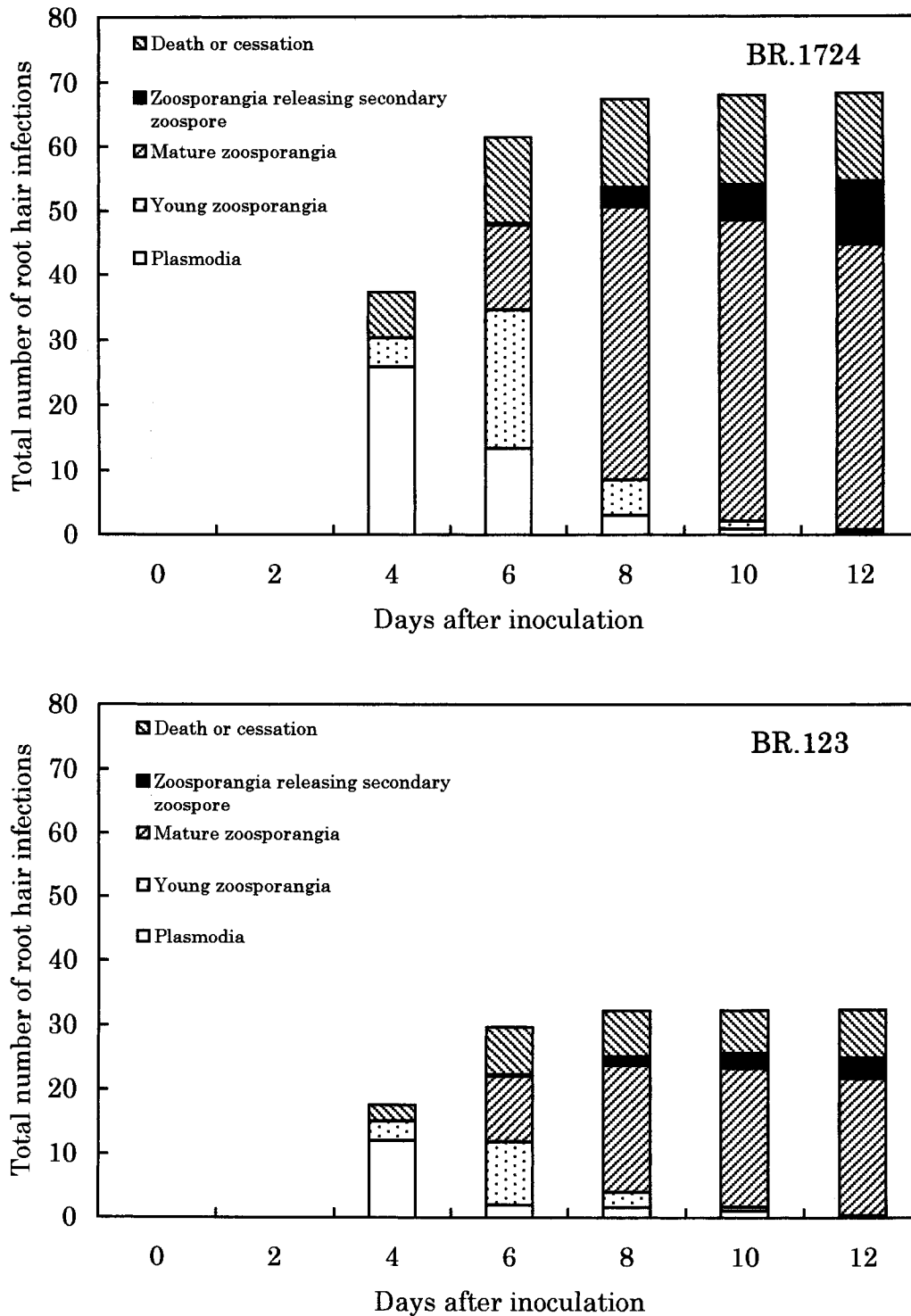

Fig. 3. Growth of *Plasmodiophora brassicae* in the root hair of turnip hairy root.

was observed in the sections formed after inoculation (days 4–6 and day 6 respectively for roots inoculated 3 d and 5 d after transfer).

Development of *Plasmodiophora brssicae* in root hair
Growth of *P. brassicae* in the root hair of hairy root was observed at 2-d intervals until 12 d after inoculation (Fig. 3). Development of *P. brassicae* was classified into five categories: i) plasmodia, ii) young zoosporangia, iii) mature zoosporangia, iv) zoosporangia releasing secondary zoospores, and v) death or cessation of growth, when cytoplasm of the pathogen agglutinates in the root hair. In strain BR. 1724, plasmodia and young zoosporangia were observed 4 d after inoculation. Most primary plasmodia differentiated to mature zoosporangia 8–10 d after inoculation. The secondary zoospores were initially released 6 d after inoculation. A similar trend was observed in strain BR. 123, although the number of root hair infections was less than in strain BR. 1724.

These development stages were also observed serially in one root hair in both strains (Fig. 4). Amorphous primary plasmodium (Fig. 4a) expanded to fill as much as one-quarter of the volume of the root hair within 1 d (Fig. 4b). The protoplasm of plasmodium became tuberculate in shape, and each tubercule subsequently became bounded by a membrane and developed zoosporangia (Fig. 4c). The cytoplasm within zoosporangia cleaved and then differentiated into mature zoosporangia containing secondary zoospores. Secondary zoospores were then released from zoosporangia (Fig. 4d).

Discussion

Several attempts have been made to enhance germination of resting spores of *P. brassicae* using root exudates and old or decaying galls (Macfarlane, 1970; Suzuki et al., 1992). However, a comprehensive understanding of the mechanism of spore germination is still lacking, probably because microbial contamination of resting spores has hindered studies. Indeed, Macfarlane (1970) noticed that microbial contaminants in resting spore suspension resulted in non-reproducibility of germination. In the present study, surface-disinfected resting spores of *P. brassicae* showed higher germination than non-disinfected resting spores. This result suggests that the germination of resting spores may be affected by microbial contaminants. Microbial contamination might affect the spore germination, for example, by causing fungistasis (Takahashi, 1994). However, it is necessary to investigate further whether microbes in the non-disinfected resting spore suspension suppress spore germination.

Previous studies (Samuel and Garrett, 1945; Naiki et al., 1978) reported that the age of root hair might affect root hair infection by *P. brassicae*. But serial observation of root hair infection by resting spores is difficult by the traditional method of soil and hydrophonic cultures. In contrast, our dual culture of hairy roots and *P. brassicae* under axenic conditions allowed easy serial observation of root hair infection in the same sample. The present study showed clearly that the age of root hair
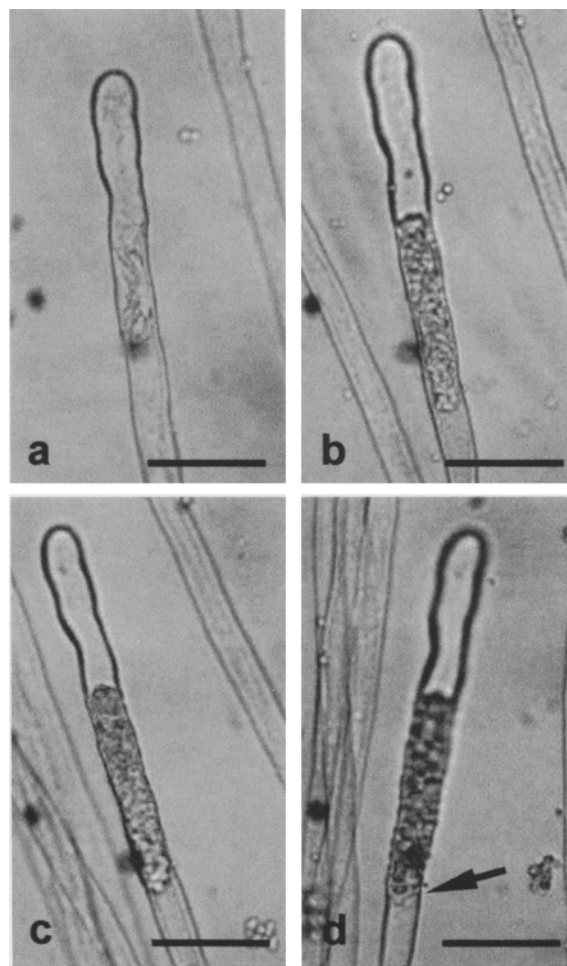

Fig. 4. Growth of *Plasmodiophora brassicae* within the same root hair of turnip hairy root photographed 4, 5, 6, and 8 d (a, b, c, and d, respectively) after inoculation with resting spores. a. Primary plasmodium. b. Grown primary plasmodium. c. Zoosporangia. d. Partly evacuated zoosporangia. Arrow indicates empty zoosporangium. Scale bars: 10 μm.

influenced the root hair infection, since the number of infections was high in the sections formed 1 d before inoculation. It is interesting to note that 5- to 6-d-old root hairs were more susceptible to infection by *P. brassicae* than younger or older root hairs.

The growth of *P. brassicae* within the root hairs of hairy root was similar to that observed in intact plants by previous workers (Katsura et al., 1970; Ingram and Tommerup, 1972). Therefore, pathogen development of *P. brassicae* in the hairy roots is apparently similar to that in intact plants, even though hairy roots without shoots may differ physiologically and biochemically from those of intact plants.

The present dual culture method has other advantages over the traditional method of using soil. Experiments can be conducted in a defined chemical and physical environment, such as temperature, humidity, pH, and nutritional conditions, without the effects of other micro-

organisms. Because we were successful in establishing gall formation in hairy root cultures in the previous study (Asano et al., 1999), this method will be applied to observe the site and timing of the second stage of infection by *P. brassicae*. Therefore, the present dual culture method will be useful for understanding the effect of environmental factors on the infection and growth of *P. brassicae*.

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