Surface Disinfestation of Resting Spores of *Plasmodiophora brassicae* Used to Infect Hairy Roots of *Brassica* spp.

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ABSTRACT

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Resting spores of *Plasmodiophora brassicae* were surface-disinfested by treatment with 2% chloramine-T for 20 min and then with an antibiotic solution (1,000 ppm of colistin sulfate, 1,000 ppm of vancomycin hydrochloride, and 6,000 ppm of cefotaxime sodium) for 1 day. The disinfested resting spores were used to inoculate hairy roots of cabbage (*Brassica oleracea* var. *capitata* cv. Fuji Wase), Chinese cabbage (*B. pekinensis* cv. Musou Hakusai), turnip (*B. rapa* var. *rapifera* cv. Wase Okabu), and rape (*B. napus* line Dc 119). Differences among hosts in

susceptibility to clubroot in hairy roots were evident. Chinese cabbage and turnip hairy roots supported the highest percentages of root hair infection (53.3 to 80%) and the greatest production of zoosporangial groups (8.5 to 32.5 per root). Moreover, gall formation was observed only on Chinese cabbage and turnip hairy roots. The morphology of zoosporangia, plasmodia, and resting spores in diseased hairy roots was found to be identical to that in infected intact plants by both light and scanning electron microscopy. Pathogenicity tests confirmed the infectivity of resting spores produced in hairy roots. Thus, the hairy root culture technique should prove useful as a dual culture system for *P. brassicae*.

Plasmodiophora brassicae Woronin, the causal agent of clubroot of crucifers, is a soilborne obligate pathogen. The fungus has never been axenically cultured, although axenic culture is a basic technique in physiological and etiological investigations of plant pathogens. Various investigations have been conducted on the use of dual cultures of *P. brassicae* with host tissue (1,2,9,10,22,23), but the propagation efficiency of *P. brassicae* was low. In addition, it is very difficult to establish and maintain axenic dual cultures because galls are infested by many microorganisms and because the quantity of resting spores recovered from infected callus is less than that from intact clubroot (9). Recently, the use of hairy root cultures, induced by infection with *Agrobacterium rhizogenes*, has received considerable attention in the study of host-parasite relationships (3,16,19). The culture of *P. brassicae* in association with hairy roots was first performed successfully by Mugnier (15) without any gall formation. Likewise, He (8) demonstrated the infection of hairy roots by *P. brassicae* using solid and liquid culture media. In a solid medium, root hair infection was observed but not gall formation; in a liquid medium, there was gall formation but not root hair infection. The galls formed in hairy roots were small, and the successful infection frequency was low (0 to 20%). Graveland et al. (6) also found small galls but did not observe *P. brassicae* resting spores in the gall tissue of hairy roots. In short, a culture system comprising hairy roots and the pathogen has not been established.

Elimination of microorganisms associated with *P. brassicae* resting spores is necessary for in vitro dual culture. In previous reports (6,8,15), resting spore suspensions were obtained from surface-sterilized galls, but such suspensions were not always disinfested. It was necessary to develop a rapid and effective technique for obtaining disinfested suspensions of resting spores as

inoculum for in vitro cultures. In this research, we developed a technique for the elimination of microorganisms from resting spore suspensions and for the establishment of an in vitro dual culture of *P. brassicae* and hairy roots of several *Brassica* spp.

MATERIALS AND METHODS

Preparation of the resting spore suspension from artificially infected clubroot. Inoculum for propagation of resting spores was prepared by the slurry method (24). The slurry consisted of 100 ml of potting soil (Guransol, Sumitono Kagaku, Osaka, Japan) and 50 ml of a resting spore suspension and had a final concentration of 1.0×10^7 spores per g. The slurry was mixed thoroughly and poured into a hole 3 cm in diameter and 2 cm deep in compost potting soil (Zeolite, Okutamakougyo Co. Ltd., Tokyo, Japan) contained in plastic pots (11 $\times$ 8 cm). Seeds of turnip (*B. rapa* L. var. *rapifera* Merz. cv. Wase Okabu) were surface-sterilized with 70% ethanol for 1 min and 10% hydrogen peroxide for 1 h and then rinsed three times in sterile distilled water. The surface-sterilized seeds were incubated for 1 day in the dark at 25°C. The seedlings were planted in the slurry-amended potting soil and grown in the greenhouse at 20 to 25°C. The galls were collected about 8 weeks after inoculation and 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, cut into small pieces, macerated in a blender in 50 ml of sterile distilled water for 3 min, and then filtered through eight layers of cheesecloth. The filtrate was centrifuged at $1,700 \times g$ for 5 min and washed five times with sterile distilled water. The spore suspension was prepared with sterile distilled water and adjusted to a concentration of 5.0×10^7 spores per ml.

Surface disinfestation of resting spores. The spore suspension was centrifuged at $1,700 \times g$ for 5 min, and the supernatant was discarded. Resting spores were suspended in freshly prepared

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2% chloramine-T solution (wt/vol) (Wako Pure Chemical Industries Ltd., Osaka, Japan) at room temperature for 20 min and then washed twice in sterile water by centrifugation as described. The resulting pellet was suspended in an antibiotic solution containing 1,000 ppm of colistin sulfate (Wako Pure Chemical Industries Ltd.), 1,000 ppm of vancomycin hydrochloride (Wako Pure Chemical Industries Ltd.), and 6,000 ppm of cefotaxime sodium (Hoechst Pharmaceuticals and Chemicals K. K., Tokyo) in distilled water and incubated at 25°C in the dark. After 1 day, the suspension was washed twice in sterile water by centrifugation. The resting spores were suspended in 1/10-strength modified Hoagland's solution (5 mM Ca[NO₃], 5 mM KNO₃, 2 mM MgSO₄, and 2 mM KH₂PO₄; pH 5.8) (12) with or without 100 ppm of cefotaxime sodium. Resting spores not treated with chloramine-T and antibiotics were used as the control treatment. The resting spore suspension was incubated in the dark at 25°C. After 10 days, drops (about 10 µl) of the spore suspension were placed on glass slides, and germinated and ungerminated resting spores were directly counted by using a Nomarski differential interference contrast optic microscope (Olympus Optical Co. Ltd., Tokyo). At least 300 spores were randomly counted per slide.

Assessment of contamination of resting spores. To determine whether or not the spore suspension was disinfested, the growth of microbial contaminants was assessed twice, first at the time that chemicals were removed from the spores and again when spore germination was assessed. To assess contamination, five droplets of the suspension were placed on plate count agar medium (Nissui Chemical Co. Ltd., Tokyo) in 9-cm petri plates and incubated at 25°C in the dark. After 1 week, the plates were visually assessed for colony formation in the droplets. This experiment was conducted three times with three replicates per experiment.

Hairy root strains. Seven hairy root strains were induced in plants from four crucifer species: cabbage, *B. oleracea* L. var. *capitata* L. cv. Fuji Wase; Chinese cabbage, *B. pekinensis* (Lour.) Rupr. (formerly *B. rapa* var. *pekinensis*) cv. Musou Hakusai; turnip cultivar Wase Okabu (Takii Seed Co., Ltd., Kyoto); and rape, *B. napus* L. line Dc 119 (Tohoku Seed Co., Ltd., Tochigi, Japan) (Table 1). All cultivars were highly susceptible to *P. brassicae*. Hairy roots were subcultured every 4 weeks on petri dishes containing Murashige and Skoog's (MS) agar medium (17).

Inoculation with *P. brassicae*. Hairy roots of *Brassica* spp. were inoculated with resting spores of *P. brassicae* by the method of Graveland et al. (6) with modifications. Hairy roots were cut into segments approximately 3 cm long, placed on MS medium in 9-cm petri dishes, and cultured in the dark at 25°C for 5 days to allow growth of root hairs. Five milliliters of disinfested resting spore suspension (5.0×10^6 spores per ml in 1/10-strength Hoagland's solution supplemented with 100 ppm of cefotaxime sodium) was put over the hairy roots, and the plates were incubated in the dark at 25°C. After 12 days, the inoculated hairy roots were observed for root hair infection in the taproot (0 to 7 cm from the basal part of the hairy root) by using an inverted microscope (Olympus Optical Co. Ltd.). The hairy roots were incubated for 7 weeks under the same conditions. The gall formation was assessed on the basis of the number of galls and fresh weight of galls per plate.

TABLE 1. Hairy root strains used in this study

Hairy root strain	Host plant	Bacterial strain ^z
BO. 123	Cabbage	ArM-123
BP. 123	Chinese cabbage	ArM-123
BP. 1724	Chinese cabbage	NIAES 1724
BR. 13	Turnip	A13
BR. 123	Turnip	ArM-123
BN. 123	Rape	ArM-123
BN. 1725	Rape	NIAES 1725

^z ArM-123, NIAES 1724 and A13, and NIAES 1725 were obtained from Shizuoka Prefectural Agricultural Experiment Station; Research Institute of Agricultural Research, Ishikawa Agricultural College; and Lion Corporation Research Institute, respectively.

The control treatment consisted of hairy roots inoculated with 5 ml of 1/10-strength Hoagland's solution amended with 100 ppm of cefotaxime sodium.

Intact seedlings of *Brassica* cultivars, in which the transformed roots had been induced, were also inoculated with resting spores. The inoculum slurry consisted of 100 ml of autoclaved soil and 50 ml of spore suspension in distilled water comprising 5.0×10^6 disinfested resting spores per gram of slurry. This slurry was mixed thoroughly and poured into a hole (3 cm in diameter and 2 cm deep) in the compost contained in a plastic pot (6 × 7.5 cm). Five 1-day-old seedlings were then transplanted to the slurry-amended compost. After a 12-day incubation period, the seedlings were washed thoroughly under running tap water to remove soil particles, and the taproots were stained with 0.005% cotton blue solution in 50% acetic acid for more than a day. Zoosporangial clusters were counted on 4 cm of each taproot from the basal part of the hypocotyl.

Eight weeks after incubation, the plants were examined for clubroot symptoms. For each plant, the disease index class was visually determined by using a scale of 0 to 3, in which 0 = no swelling visible; 1 = slight swelling, usually confined to lateral roots; 2 = moderate swelling on lateral or taproots, and 3 = severe swelling on lateral or taproots. The number of plants in class 0 was multiplied by 0, in class 1 by 20, in class 2 by 80, and in class 3 by 100, and sum was divided by the total number of plants to give a disease severity for each treatment (18). Fresh weights of roots with galls were also recorded. Five seedlings were planted in each pot. Two pots were used for root hair infection and zoosporangial enumeration, and three pots were used for assessment of gall formation and disease severity. The experiment was conducted twice.

Histology of *P. brassicae* in hairy roots. To observe root hair infection, the hairy roots were washed under tap water, stained as described above, and then examined with a light microscope. For observation of secondary plasmodia and resting spores, the sections of gall tissues were stained with 0.05% cotton blue solution in 50% acetic acid, and specimens were examined by light microscopy.

For scanning electron microscopy, galls derived from hairy roots were cut into approximately 3-mm pieces. The tissues were fixed in 5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2) at room temperature for 4 h and washed four or five times with fresh buffer. The fixed tissues were postfixed with 1% osmic acid in the same buffer at room temperature for 2 h and then rinsed four or

TABLE 2. Effects of several chemicals on the suppression of microorganisms and germination of resting spores of *Plasmodiophora brassicae*

Treatment ^u	Cefotaxime sodium ^v	Contamination ^w (%)		Germination ^x (%)
		0 days	10 days	
Control	—	100.0 b ^y	100.0 b	15.8 abc
	+	71.1 b	100.0 b	16.0 abc
Chloramine-T	—	0.0 a	68.9 b	8.1 a
	+	0.0 a	68.9 b	15.8 abc
Antibiotics ^z	—	6.7 a	0.0 a	19.4 bc
	+	11.1 a	0.0 a	27.0 c
Chloramin-T + antibiotics	—	0.0 a	0.0 a	9.8 ab
	+	0.0 a	0.0 a	15.1 abc

^u Resting spores were suspended in 2% chloramine-T for 20 min, antibiotic solution for 1 day, or a combination of the two.

^v After treatment with chloramine-T and antibiotics, resting spores were suspended in 1/10-strength Hoagland's solution with (+) or without (—) 100 ppm of cefotaxime sodium.

^w Percentage of contamination was assessed at the time of treatment with chemicals and 10 days after treatment. Data are the means of three trials, each with three petri plates.

^x Germination of resting spores was assessed with a Nomarski interference optic microscope. Values are means of three replicates of samples with more than 300 spores per sample of spore suspension.

^y Means within a column followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

^z Colistin sulfate (1,000 ppm), vancomycin hydrochloride (1,000 ppm), and cefotaxime sodium (6,000 ppm).

Fig. 1. Gall formation on turnip hairy roots 8 weeks after inoculation with resting spores of *Plasmodiophora brassicae*. **A**, In vitro mass propagation of galls on Murashige and Skoog's medium. Bar = 20 mm. **B**, Galls shown at high magnification. Bar = 10 mm.

five times with the buffer to remove excess osmic acid. The double-fixed samples were dehydrated through an ethanol series of 25, 50, 70, 90, 95, 100, and 100% for 15 min each. The ethanol was removed by changes of ethanol and amyl acetate at 1:3, 1:1, and 3:1 and two changes of 100% amyl acetate for 25 min each. The specimens were critical-point dried, mounted on specimen studs, and sputter coated with gold palladium. Samples were examined with a scanning electron microscope (model JSM-U3; Japan Electron Optics, Tokyo) operated at 10 kV.

Pathogenicity of resting spores derived from galls in hairy roots. Resting spores from galls developed in hairy root cultures after 8 weeks of incubation on MS medium were used to inoculate susceptible turnip seedlings ('Wase Okabu') by the slurry method described above. The slurry consisted of 100 ml of autoclaved soil and 50 ml of the spore suspension in distilled water at a concentration of 1.0×10^6 disinfested resting spores per gram of slurry. This slurry was mixed thoroughly and poured into a hole (3 cm in diameter and 2 cm deep) in the compost contained in a plastic pot (11 $\times$ 8 cm). One-day-old turnip seedlings were then transplanted to the slurry-amended compost and grown in the greenhouse. Seedlings grown in compost soil amended with slurry lacking resting spores were used as the uninoculated control treatment. After 8 weeks, the seedlings were assessed for clubroot symptoms. The experiment was conducted twice with five replicates per experiment.

Statistical analysis. Each experiment had a completely randomized design with two, three, or five replicates per treatment. Percentage data were transformed by square root or arcsine transformations to stabilize the variance (4). The treatment means were separated by Duncan's multiple range test ($P < 0.05$).

RESULTS

Surface disinfestation of resting spores. Resting spores treated with chloramine-T showed no contamination at the time of the treatment, but a high percentage of contamination was found 10 days after treatment (Table 2). Exposing the spores to antibiotics did not eliminate any bacteria at the time of treatment. The combination of chloramine-T and antibiotics completely eliminated contamination even 10 days after treatment, and the treated spores were as germinable as those of the untreated controls. To prevent reinfection of resting spores during incubation, cefotaxime sodium was added to the spore suspension. The addition did not affect the effectiveness of disinfestation or the germinability of resting spores.

Infection of hairy roots. Root hair infection was observed in all seven strains of hairy roots, but the frequencies differed among the host plant species (Table 3). Chinese cabbage and turnip hairy roots showed up to 80.0% incidence of root hair infection. In contrast, the percentages of root hair infection in cabbage and rape hairy roots were moderate and low, respectively. Similar trends were observed with the number of zoosporangial groups. Chinese cabbage and turnip hairy roots developed galls 8 weeks after

TABLE 3. Root hair infection and gall formation in hairy roots inoculated with *Plasmodiophora brassicae*^x

Hairy root strain ^y	Root hair infection		Gall formation		
	Infection (%)	Zoosporangial groups/infected hairy root (no.)	Infection (%)	Galls/infected hairy root (no.)	Gall fresh weight/infected hairy root (mg)
BO. 123	6.7 a ^z	0.3 a	0.0 a	0.0 a	0.0 a
BP. 123	73.3 b	18.5 ab	33.3 ab	39.9 a	14.8 a
BP. 1724	80.0 b	22.0 ab	13.3 ab	18.3 a	3.8 a
BR. 123	53.3 ab	8.5 a	46.7 b	276.6 b	312.3 b
BR. 13	80.0 b	32.5 b	20.0 ab	75.2 a	164.3 ab
BN. 123	26.7 ab	1.9 a	0.0 a	0.0 a	0.0 a
BN. 1725	46.7 ab	4.1 a	0.0 a	0.0 a	0.0 a

^x Values are means of three trials, each with five replicates. Resting spores were obtained from the galls 8 weeks after inoculation.

^y BO., BP., BR., and BN. = cabbage, Chinese cabbage, turnip, and rape, respectively.

^z Means within a column followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

inoculation, while cabbage and rape hairy roots showed no gall formation (Table 3). The number and fresh weight of galls formed in turnip hairy roots were both greater than those of Chinese cabbage hairy roots. The galls in turnip hairy roots were spherical or spindle shaped and were scattered throughout the hairy roots (Fig. 1). Root hair infection and gall formation were not observed in uninoculated controls.

Microscopic observations. There were no differences in the structure of zoosporangia among the seven hairy root strains. All stages in root hair infection were observed, including the development of immature zoosporangia and complete evacuation of zoosporangia (Fig. 2). The morphology of zoosporangia and the growth stages of root hair infection in hairy root cultures were identical to the morphology and growth stages in infected intact plants.

The secondary plasmodia and immature resting spores within whitish gall tissues were observed by light and electron microscopy 6 weeks after inoculation. Eight weeks after inoculation, however, galls became brownish, and a number of mature resting spores were observed in the gall tissues (Fig. 3). The cortical in-

fection phase of the pathogen was also identical in hairy roots and diseased plants grown in infested soil.

Pathogenicity of resting spores from diseased hairy roots.

The pathogenicity of resting spores from 8-week-old galls in hairy roots was tested on turnip seedlings by the slurry method (Table 4). Resting spores from galls developed in hairy root cultures showed the same infectivity as those from infected intact plants. All inoculated plants had shown severe clubroot symptoms.

Susceptibility of the intact plant. Root hair infection was observed in all host species (Table 5). The percentage of infected plants and the number of zoosporangial groups per root were not significantly different among host plants. All four plant species also formed galls with high disease severity.

DISCUSSION

There have been several studies of dual cultures of obligate parasites (5,7,21). However, dual-culture techniques are not common. Obtaining surface-disinfested spores from infected plants has been

Fig. 2. Root hair of a turnip hairy root 12 days after inoculation with *Plasmodiophora brassicae*. **A**, Immature zoosporangia in the root hair; **B**, zoosporangia containing zoospores in the root hair; **C**, partially evacuated zoosporangia in the root hair; and **D**, completely evacuated zoosporangia in the root hair. Bars = 20 μ m.

a major technical problem. Sacristan and Hoffmann (20), Mugnier (15), and He (8) obtained disinfested resting spore suspensions of *P. brassicae* by surface sterilization of whitish clubroot galls. In preliminary experiments for this study, however, the previously reported methods were not effective in the elimination of microorganisms. Mertz et al. (13) and Mosse (14) obtained axenic spores in arbuscular mycorrhizal fungi with direct treatment using a disinfectant solution. In the current study, disinfectants were directly

applied to the resting spores for elimination of microorganisms. Incubation of *P. brassicae* resting spores in 2% chloramine-T for 20 min and then in antibiotics for 1 day prevented the multiplication of microbes without affecting germination.

We were successful in establishing root hair infection and gall formation in hairy root cultures. Compared with the results of He (8) and Graveland et al. (6), the percentages of gall formation and the number of galls were much higher in the current study. Two

TABLE 4. Pathogenicity of resting spores of *Plasmodiophora brassicae* to turnip seedlings

Resting spore source ^w	Viability of resting spores ^x (%)	Clubroot	
		Incidence (%)	Disease severity ^y
Hairy root	79.3 a ^z	100.0 b	100.0 b
Intact plant	77.5 a	100.0 b	100.0 b
Control	...	0.0 a	0.0 a

^w Resting spores were obtained from the galls formed on hairy roots or diseased intact plants 8 weeks after inoculation.
^x Viability of resting spores was assessed by Nomarski interference optic microscopy.
^y Disease severity = [(N0 × 0) + (N1 × 20) + (N2 × 80) + (N3 × 100)] / (N0 + N1 + N2 + N3). N0, N1, N2, and N3 are the numbers of seedlings in disease index classes 0 to 3, respectively. Disease index classes: 0 = no clubroot; 1 = slight swelling; 2 = moderate swelling on lateral or taproots; and 3 = severe swelling on lateral or taproots.
^z Means within a column followed by the same letter are not significantly different (*P* < 0.05) according to Duncan's multiple range test.

TABLE 5. Root hair infection and gall formation on several *Brassica* plants^x

Host plant	Root hair infection		Gall formation		
	Incidence (%)	Zoosporangial groups (no.)	Incidence (%)	Disease severity ^y	Fresh wt (g)
Cabbage	100.0 a ^z	100.2 a	100.0 a	100.0 a	0.9 a
Chinese cabbage	100.0 a	53.8 a	100.0 a	100.0 a	1.4 b
Turnip	100.0 a	86.6 a	100.0 a	100.0 a	2.2 c
Rape	100.0 a	81.2 a	100.0 a	100.0 a	1.8 bc

^x Values are means of two trials. Two replicates were used for the root hair infection experiment, and three replicates were used for the gall formation experiment.
^y Disease severity = [(N0 × 0) + (N1 × 20) + (N2 × 80) + (N3 × 100)] / (N0 + N1 + N2 + N3). N0, N1, N2, and N3 are the numbers of seedlings in disease index classes 0 to 3, respectively. Disease index classes: 0 = no clubroot; 1 = slight swelling; 2 = moderate swelling on lateral and/or taproots; and 3 = severe swelling on lateral and/or taproots.
^z Means within a column followed by the same letter are not significantly different (*P* < 0.05) according to Duncan's multiple range test.

Fig. 3. A and C, Light micrographs and B and D, scanning electron micrographs of gall tissue of turnip hairy roots inoculated with *Plasmodiophora brassicae*. A and B, 6 weeks after inoculation. Secondary plasmodia can be seen in the gall tissue. C and D, 8 weeks after inoculation. Resting spores have developed in the gall tissue. Bars = 20 µm.

factors contributed to the differences. First, using a disinfested resting spore suspension allowed an effective use of more resting spores (5×10^6 spores in 5 ml) for inoculation. The use of more resting spores may have increased the chance of infection, resulting in root hair infection and gall formation in lateral roots as well as taproots (data not shown). A second factor was the use of different host plant species. In the current study, susceptibility of hairy roots differed among plant species. Higher percentages of root hair infections were obtained in Chinese cabbage (*B. pekinensis*) and turnip (*B. rapa*) hairy roots. Furthermore, no gall formation was observed in cabbage and rape hairy roots belonging to *B. oleracea* and *B. napus*, respectively. *B. pekinensis* and *B. rapa* were not used in previous studies (6,8,15). In those experiments, the hairy roots were induced in *B. oleracea* and *B. napus*.

Although all plant species used in our work were highly susceptible to *P. brassicae*, there were differences in susceptibility among the strains of hairy roots, probably resulting from differing culture conditions between inoculations of intact host plants and hairy roots. In inoculations of hairy roots, environmental factors, culture media, or moisture conditions were not optimal for the development of clubroot disease. In fact, Chinese cabbage and turnip hairy roots grew vigorously and rapidly under the conditions of our research, but cabbage and rape hairy roots grew more slowly. Therefore, galls formed in Chinese cabbage and turnip hairy roots but not in cabbage and rape hairy roots. Another possibility is that the physiological characteristics of hairy roots might not always be identical to those of intact roots and that the responses of hairy roots to the clubroot pathogen might be different from those of intact roots. Isa et al. (11) reported that water-soluble mucilages produced by hairy roots of *Astragalus gummifer* were not the same as those of the mother plant.

Turnip hairy roots will be more useful for dual cultures of *P. brassicae* than those of Chinese cabbage, because they formed large and numerous galls. This difference in gall formation between turnip and Chinese cabbage hairy roots is probably a reflection of the intact plant's properties. In short, the clubbed roots of intact turnip were enlarged, whereas those of intact Chinese cabbage were slender. Furthermore, symptoms exhibited by the diseased roots of turnip were more severe than those of Chinese cabbage.

The hairy root culture technique for *P. brassicae* has obvious advantages over callus culture systems because it permits observation of fungal development in vitro. The spores formed in hairy root cultures are viable and can infect intact host plants. In contrast, callus cultures support poor growth of *P. brassicae* (9), and resting spores are not easily obtained. However, continuous cultures have not yet been achieved by the hairy root culture technique. Continuous cultures on agar medium are a common technique for maintaining and propagating facultative parasites and saprophytes. Thus, it would be highly desirable to develop a continuous subculture technique in association with hairy roots in vitro.

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