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Running title: *Inhibition of* Plasmodiophora brassicae *spore germination*

Suppression of clubroot disease under neutral pH caused by inhibition of spore germination of *Plasmodiophora brassicae* in the rhizosphere

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To elucidate the mechanism of clubroot suppression under neutral soil pH, a highly

reproducible germination assay system under soil culture conditions was designed based

on the hypothesis that germinated spores of *Plasmodiophora brassicae* could be

identified by the absence of a nucleus (i.e. having released a zoospore to infect a root

hair of the host plant). *Brassica rapa* var. *perviridis* seedlings were inoculated with a

spore suspension of *P. brassicae* at a rate of 2.0×10^6 spores g^{-1} soil and grown in a

growth chamber for 7 days. The spores were recovered from rhizosphere and non-rhizosphere soils and stained with both Fluorescent Brightener 28 (cell-wall-specific) and SYTO 82 orange fluorescent nucleic-acid stain (nucleus-specific stain). Total numbers of spores were counted under UV-excitation, and spores with a nucleus that fluoresced orange under G-excitation were counted. The significant increase in the percentage of spores without a nucleus (germinated spores) in the rhizosphere after 7 days' cultivation and the correlation with root-hair infections validated the assay system. Applications of calcium-rich compost or calcium carbonate to neutralize the soil significantly reduced the percentage of germinated spores in the rhizosphere, as well as the number of root-hair infections. The present study provides direct evidence that the inhibition of spore germination is the primary cause of disease suppression under neutral soil pH.

Keywords: *Brassica rapa*, nuclear/cell-wall double staining, rhizosphere, soil pH, soilborne disease, spore germination

Introduction

Clubroot of cruciferous plants caused by the plasmodiophoromycete *Plasmodiophora*

brassicae, is a major disease that is widespread throughout the world. The life cycle of *P. brassicae* consists of two phases: the primary phase is characterized by germination of resting spores and subsequent infection of root hairs, and the secondary phase is characterized by the colonization and proliferation of the pathogen in the root cortex (Ingram & Tommerup, 1972). It is difficult to control the disease because the pathogen survives in soil for a long time as resting spores (Wallenhammar, 1996). Liming has been employed as a traditional and conventional technique to control the disease, and the disease-suppressive effects of the neutralization of soil acidity are well documented (Colhoun, 1953; Dobson *et al.*, 1983; Campbell *et al.*, 1985; Murakami *et al.*, 2002; Tremblay *et al.*, 2005). Attempts to differentiate the effects of calcium from those of pH have been carried out. Webster & Dixon (1991) employed the sand-solution culture system to investigate the effects of pH on primary (root-hair) infection and clubroot development in the absence and presence of calcium. They observed independent and synergistic effects of pH and calcium on the suppression of zoospore invasion, zoosporangial maturation and clubroot development. Disease-suppressive soils were found in the field where large amounts of calcium-rich organic matter, either farmyard manure or food factory sludge compost, had been incorporated for more than 15 years (Niwa *et al.*, 2007). In these soils, it was demonstrated that an increase in soil pH as a

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result of the accumulation of calcium was the primary cause of the suppression of

root-hair infection and that the effect of calcium was additive, and limited under neutral

conditions. Although the suppression mechanism of soil neutralization and that of

calcium enrichment need to be elucidated comparatively, all these observations confirm

that the occurrence of clubroot disease is suppressed effectively under neutral soil pH in

the presence of calcium, leading to the hypothesis that either spore germination or

root-hair infection, each a part of the primary phase of the life cycle of the pathogen, is

inhibited under such conditions.

To test this hypothesis requires a reliable assay system to examine germination of

resting spores under soil culture conditions. Naiki *et al.* (1987) demonstrated that

germinated spores, i.e. those that had released a zoospore, could be distinguished from

ungerminated spores using Nomarski differential interference contrast microscopy. This

technique was applied to a resting-spore/Ri-T-DNA-transformed-root dual-culture

system that enabled the observation of germination and subsequent infection of the

roots on the same plate (Asano *et al*., 2000). Application of this assay system, however,

is limited to *in vitro* cultures, because any contamination of soil particles or

microorganisms seriously interferes with evaluation. By contrast, Takahashi &

Yamaguchi (1989) recovered spores after incubation in soil and stained them with

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Fluorescent Brightener 28 (= calcofluor white, a cell-wall stain) and ethidium bromide (a cell-impermeable nucleic-acid stain), which showed blue and red fluorescence under UV-excitation, respectively. They claimed that the ethidium-bromide-stained (red-fluorescent) spores were inactive because the dye was impermeable to live cells. Furthermore, Takahashi (1991) incubated spores in the presence and absence of host plants and found a correlation between the numbers of ethidium-bromide-stained and germinated spores using both fluorescent and Nomarski differential interference contrast microscopy. Spores stained with ethidium bromide, however, might include not only germinated spores, but also damaged spores incapable of germinating (Takahashi, 1991), so ethidium bromide might be unsuitable for the detection of germinated spores. By contrast, the detection of ungerminated spores retaining a zoospore (with a nucleus) seemed more promising, and it was considered that a cell-permeable nucleic-acid stain could be used for this purpose.

The objective of this study was to clarify the mechanism of disease suppression at neutral soil pH in the assessment of spore germination. The reliability and reproducibility of the germination assay system were examined in conjunction with the soil-based plug-pot bioassay (Niwa *et al*., 2007), with particular emphasis on the validity of nuclear staining for the germination assay. It was uncertain, however,

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whether the assay system was sensitive enough to detect germination, because if spores

germinated in response to root exudates (Macfarlane, 1970; Suzuki *et al*., 1992), only a

small proportion of spores in the vicinity of roots would germinate. To confirm this,

spores were extracted both from rhizosphere and non-rhizosphere soils separately after

culturing with host plants.

Materials and methods

Inoculum preparation

Clubroots (galls) infected with *Plasmodiophora brassicae* formed on chinese cabbage

(*Brassica rapa* var. *pekinensis* cvs Satokaze and Tomikaze) were obtained from the

experimental field of Nagoya University Togo-cho, Aichi, Japan (Niwa *et al*., 2007) and

stored at -20˚C. The frozen galls were thawed in water, washed thoroughly under

running tap water, surface-sterilized with 70% (v/v) ethanol for 30 s, homogenized in

sterilized water and filtered through eight layers of cheesecloth. The filtrate was

centrifuged at 2580 *g* for 5 min. The pellet was washed four times with sterilized water

and then resuspended in a known volume of sterilized water. A 100-µL aliquot of the

suspension was mixed with an equal volume of 200 µg mL^{-1} Fluorescent Brightener 28

(Sigma-Aldrich) solution (Takahashi & Yamaguchi, 1987). Numbers of resting spores

were counted in a Thoma blood corpuscle chamber using fluorescence microscopy with

UV-excitation at ×200 magnification (Olympus BX51).

Glass-bead solution culture and germination assay by nuclear staining

Fifty seeds of *B. rapa* var. *perviridis* cv. Komatsuna Rakuten were surface-sterilized

with 70% ethanol for 30 s and 10% sodium hypochlorite (available chlorine 5%) (w/v)

containing 0.01% (v/v) Tween 20 for 5 min, then rinsed in sterilized water. The seeds

were sown on 20 g of autoclaved glass beads (0.35-0.5 mm diam) moistened with 4 mL

of filter-sterilized 1 mL L^{-1} 6-10-5 HYPONeX and 200 µg mL^{-1} streptomycin (Wako

Pure Chemical) in a 6-cm Petri dish and incubated at 25˚C in the dark. After 2 days, the

seedlings were inoculated with 2×10^6 spores of *P. brassicae* suspended in 2 mL

sterilized water and further incubated under similar conditions for 5 days. The spores

were also incubated in the absence of host plants under the same conditions as the

control. The medium and seedlings in the Petri dish were transferred to a 200-mL

beaker by washing with 0.02% (v/v) Tween 20 and filtered through a 37-µm nylon mesh

to separate the spores from the plants and glass beads. The volume of filtrate was

reduced after centrifugation, achieving a final spore density of 5000-10 000 spores µL^{-1}.

A 100-µL aliquot of the spore suspension was mixed with an equal volume of

2-µg mL^{-1} 4'-6-diamidino-2-phenylindole-dihydrochloride (DAPI) (Sigma-Aldrich) in

0.1% (v/v) dimethylformamide, and 10 µL of the mixture were transferred onto a glass

slide and covered with an 18- × 18-mm cover glass. Total numbers of spores and the

number of spores with a nucleus in the same field were counted in the bright field and

under UV excitation, respectively, using the fluorescent microscope at ×400

magnification. Approximately 100 spores were examined from each specimen (from

each Petri dish). Three Petri dishes were employed for each treatment ($n = 3$). The

experiment was conducted twice independently to confirm reproducibility.

Soil culture system and germination assay by nuclear/cell-wall double staining

Plug-pot soil culture system and sampling

A disease-conducive soil was collected from the experimental field of Nagoya

University (Niwa *et al*., 2007), air-dried in a greenhouse for a few days, passed through

a 5-mm sieve and stored at room temperature. The chemical properties of the soil were

as follows: total C, 23.4 g kg^{-1}; total N, 2.5 g kg^{-1}; total P, 5.4 g kg^{-1}; exchangeable Ca^{2+},

65.0 mEq kg^{-1}. The soil was mixed with the *P. brassicae* spore suspension at 2.0×10^6

spores g^{-1} soil and divided into two portions: one was stored at 4˚C for 1 day as the

no-plant control, and the other was incubated in the presence of host plants as follows.

Nine seeds of *B. rapa* var. *perviridis* were sown on the soil in each 4.5-cm-square plug

pots (70-mL volume) and the pots placed in the growth chamber (25°C, 16/8 h

light/dark). The plants were irrigated via the drainage hole of the pot using a capillary

sheet and were thinned to four plants per pot 4 days after sowing. Seven days after

sowing, the roots were carefully excised from the medium and sonicated at 24 Hz for 30

s in 40 mL distilled water in a 50-mL glass beaker to separate the adhering *P. brassicae*

spores and soil (0.5-1.0 g per plant, 2-4 g in total) from the roots. The roots were stained

with 50 mg L^{-1} aniline blue in 5 g L^{-1} phenol solution for 24 h at room temperature, and

the number of zoosporangial clusters of *P. brassicae* formed in root hairs of the top 2

cm of tap root was counted under a microscope at ×100 magnification. The spore/soil

suspension was transferred to a 50-mL plastic tube and centrifuged at 1120 *g* for 20 min

at room temperature. The resulting pellet was designated the 'rhizosphere soil', whereas

the rest of the medium was designated the 'non-rhizosphere soil'.

Spore extraction and germination assay

To extract *P. brassicae* spores from the soils, the rhizosphere soil (2-4 g) or the

non-rhizosphere soil (1 g) was suspended in 5 mL Percoll (GE Healthcare Bio-Sciences)

in a 50-mL plastic tube, shaken by a Cute Mixer CM-1000 (EYELA) at 1500 r.p.m. for

30 min and centrifuged at 1970 *g* for 10 min. The supernatant was transferred to a

50-mL centrifuge tube and the pellet resuspended in 5 mL Percoll and centrifuged again.

The supernatant was mixed with 40 mL distilled water and centrifuged at 1970 *g* for 20

min, and the resultant supernatant was discarded. The pellet was resuspended in distilled

water, transferred to a 15-mL centrifuge tube, washed twice with distilled water after

centrifugation at 640 *g* for 10 min and resuspended in a small amount of distilled water.

The spore suspension was transferred to a 1.5-mL tube, and the supernatant discarded

after centrifugation at 13 200 *g* for 4 min. The purified spores were fixed in 100 μL of

50% ethanol for 15 min at room temperature, centrifuged at 13 200 *g* for 4 min, washed

twice with sterilized water and resuspended in 100 μL sterilized water. A 24.75-μL

aliquot of the suspension was mixed with an equal volume of 200 μg mL^{-1} Fluorescent

Brightener 28 (a cell-wall-specific stain) solution and 0.5 μL of 5 mM SYTO 82 orange

fluorescent nucleic-acid stain (a nucleus-specific stain; Molecular Probes) in

dimethylsulfoxide and incubated at room temperature in the dark for 30 min. Ten μL of

spore suspension were transferred onto a glass slide and covered with a cover glass. The

total number of spores and the number of spores with a nucleus were counted under

UV- (330-385 nm) and G-excitations (520-550 nm), respectively, in the same field

using a fluorescent microscope at ×400 magnification. Approximately 200 spores were

observed per specimen. Three pots were employed for each treatment ($n = 3$), and the

experiment was conducted twice independently to confirm reproducibility.

   Prior to the germination assay in the soil culture system, the efficiency of the

Percoll-based method at extracting spores from the soil was evaluated as follows. The

spores prepared from the clubroots were stained (labelled) with Fluorescent Brightener

28 as described for inoculum preparation, mixed with the soil at 10^6 spores g^{-1} soil then

extracted from 1 g of soil ($n = 3$). The number of labelled spores in the extract was

counted under UV-excitation, and the percentage of recovered spores was determined as

92.6±5.8%.

Effect of calcium-rich organic matter and calcium carbonate on spore germination

Food factory sludge compost (FSC) with a particle size <1 mm was prepared as

described in Niwa *et al.* (2007). The chemical properties of FSC were as follows: total

C, 325 g kg^{-1}; total N, 46.0 g kg^{-1}; total P, 54.1 g kg^{-1}; total Ca, 91.1 g kg^{-1}; pH, 7.3. The

FSC or calcium carbonate (CaCO$_3$) was mixed with the disease-conducive soil at

25 g kg^{-1} soil (corresponding to 3.3 t ha^{-1}) or 2.5 g kg^{-1} soil (corresponding to 330 kg

ha^{-1}), and *P. brassicae* spore germination and root-hair infection were assessed

according to the method above. The pH (soil:H$_2$O, 1:2.5, w/v) of these media was

measured after harvest.

Statistics

The STATVIEW software (SAS Institute Inc.) was used for all statistical analyses. Analysis of variance (ANOVA) and Fisher's protected least significant difference test as a *post hoc* test of ANOVA ($P<0.05$) were applied for analyses of the results obtained from the spore germination assays.

Results

Spore-germination assays

Spore germination of *P. brassicae* was first assessed by nuclear staining with DAPI in glass-beads solution culture. The absence or presence of a nucleus in a spore could be discriminated under UV-excitation (Fig. 1). In the two independent experiments, the mean percentage of *P. brassicae* spores without a nucleus were 46.3 and 59.2%, respectively, before incubation, and increased significantly to 91.9 and 79.8%, respectively, after incubation in the presence of host plants (Fig. 2). The percentages of spores without a nucleus did not increase in the absence of host plants (49.1 and 55.1%, respectively). However, no root-hair infection of *P. brassicae* was observed in these

experiments. Based upon these results, it was considered that the incubation of spores in the presence of the host plant resulted in the loss of nuclei as a result of germination and that the loss of nuclei could be a potential index of spore germination. However, the nuclear-staining method with DAPI was not applicable to the soil culture system because of the non-specific staining of soil particles and other microorganisms similar to *P. brassicae* nuclei in size.

In combination with rhizosphere soil fractionation, nuclear/cell-wall double staining enabled clear discrimination of spores with or without a nucleus (Fig. 3). In two independent experiments, the mean percentage of spores without a nucleus were significantly higher in the rhizosphere soil (41.4 and 46.1%) than in the non-rhizosphere soil (25.1 and 26.2%), which gave similar values to the no-plant control (22.8 and 25.7%) (Fig. 4). Root-hair infection was successfully observed in these experiments, and the numbers of root-hair infections were 386 and 94.7 per plant in the first and second experiments, respectively.

Effect of calcium-rich organic matter and calcium carbonate on spore germination

The application of the FSC to disease-conducive soil increased the pH from 6.0 to 6.9 and the number of root-hair infections significantly decreased (Fig. 5a). In the absence

of FSC, the mean percentage of *P. brassicae* spores without a nucleus in the rhizosphere

soil was 35.9%, significantly higher than that in the non-rhizosphere soil (14.2%). By

contrast, the application of FSC reduced the mean percentage in the rhizosphere soil to

13.2%, similar to the value for the non-rhizosphere soil (10.5%). The application of

$CaCO_3$ to disease-conducive soil gave similar results (Fig. 5b). Soil pH was increased

from 6.2 to 7.1, and the number of root-hair infections decreased significantly. The

application of $CaCO_3$ significantly reduced the percentage of *P. brassicae* spores

without a nucleus in the rhizosphere soil to 15.8%, from 37.0% in controls.

Discussion

This study provides the first direct evidence that the spore germination of *P. brassicae*

in the rhizosphere is inhibited under neutral conditions created by the application of

calcium-rich organic matter or $CaCO_3$. Myers & Campbell (1985) demonstrated that

neutralization by calcium application did not reduce the viability of the resting spores,

suggesting that the effects were not fungicidal, but fungistatic to the pathogen. Webster

& Dixon (1991) suggested that an increase in soil pH by calcium application might not

eradicate the pathogen directly, but create unfavorable conditions affecting the infection

processes, e.g. enhancing host resistance to infection. The present results clarify a part

of the mechanism underlying the inhibition of the disease under neutral soil pH and raise the new question of how spore germination is inhibited under these conditions. It has been suggested that root exudates stimulate spore germination (Macfarlane, 1970; Suzuki *et al*., 1992). It is hypothesized that soil pH may influence the response of *P. brassicae* spores to root exudates or that qualitative and/or quantitative changes in the exudates may occur under neutral conditions. Identification of the germination-stimulating factors in exudates would be an important step in understanding this mechanism. It is noteworthy that stimulatory factors were released not only from cruciferous plants, but also from, for example, perennial ryegrass (Friberg *et al.*, 2005) and pearl millet (Niwa & Ezawa, unpublished results).

It was demonstrated that only spores in the rhizosphere were involved in infection. The rhizosphere is defined as the soil adjacent to roots, with different physical, chemical and biological properties than non-rhizosphere soil (Bowen & Rovira, 1999). Root activities, e.g. exudation of organic compounds and enzymes, respiration and detached root tissue, are the driving force for maintaining the environment and interactions between microorganisms (Hinsinger *et al.*, 2005). Rhizosphere soil is typically within 1-3 mm of the root surface (Mengel *et al.*, 2001), probably less for juvenile plants. The number of germinated spores in the rhizosphere of assay plants in the present

experimental system was calculated based on the amount of recovered rhizosphere soil

(0.5 g per plant) and the increase in the percentage of spores without a nucleus after 7

days of incubation (15-20%). Approximately $0.7\text{-}2.2 \times 10^5$ spores per plant germinated,

corresponding to only 0.05-0.15% of the total number of spores (1.4×10^8) in each pot.

These results suggest difficulty in controlling clubroot disease in the field. It is known

that a reduction in disease severity by decoy plants is difficult to achieve in the field,

although decooy plants effectively reduced disease severity in pot experiments by

stimulating spore germination (Murakami *et al*., 2000; Friberg *et al*., 2006). Given that

the relative volume of rhizosphere soil is much smaller in the field than in pot

experiments and only a small proportion (15-20% in the present case) of spores

germinate in the rhizosphere, major technical breakthroughs will be required to apply

this strategy to the field.

The application of food factory sludge compost (FSC) to soil increased soil pH,

calcium, carbon, nitrogen and phosphorus levels, and there could be additional effects

of these elements on disease suppression. However, this is unlikely because of the fact

that the suppressive effect in FSC-treated soils was cancelled out by acidification with

sulphuric acid (Niwa *et al*., 2007).

A highly reproducible resting spore germination assay system was developed in

combination with the plug-pot bioassay by using nuclear/cell-wall double staining technique coupled with a high recovery rate of spores extracted and purified from rhizosphere and non-rhizosphere soils.

High-resolution images of *P. brassicae* nuclei were obtained using DAPI because of its high specificity to DNA, but Fluorescent Brightener 28, a cell-wall-specific stain (Takahashi & Yamaguchi, 1987), was also needed to identify *P. brassicae* spores in the extracts prepared from soil, even after purification by the Percoll method. However, using DAPI with Fluorescent Brightener 28 is not practical because the maximal emission wavelengths of these stains under UV-excitation are very close to each other, viz. 461 and 450 nm, respectively, and thus it is difficult to assess the presence of nuclei in the spores with the two stains. SYTO 82 is a nucleic-acid stain with a maximal emission wavelength at 560 nm under G-excitation and thus does not overlap with emission from Fluorescent Brightener 28. Furthermore, SYTO 82 provides rather diffuse images of the nucleus and cytoplasm, probably because of the broad specificity of the stain, which may react with RNA in the cytoplasm as well as DNA in the nucleus (Haugland, 2002), and this supports the identification of the (ungerminated) spores under G-excitation.

Buczacki & Ockendon (1978) used 2% Calgon and 40% sucrose solution to extract

and purify spores from soil. This procedure, however, requires a few days and probably causes physiological damage to spores because of the high osmotic pressure of the sucrose solution. This is an obstacle if the viability of spores is to be assessed. Takahashi & Yamaguchi (1987) developed a method to remove the coarse particles of soil and humic substances from the spore suspension by sieving, but it was difficult to eliminate the fine particles which interfered with the identification of the spores under the microscope. The high-density centrifugation medium, Percoll, enables the extraction of the spores more conveniently and reduces fine contaminants drastically, with a high recovery rate. Removing soil particles from the spore suspension greatly improved the reliability and reproducibility of the bioassay. However, recovery of spores from organic-matter-rich soil by this method was difficult compared with that from the mineral soil used in this study (unpublished data). It is likely that spores are adsorbed onto the surface of organic matter, and improvement of the recovery rate by shaking with a detergent and/or sonication to separate the spores from organic matter is being examined.

The reproducibility of the plug-pot soil culture system was good enough to obtain similar results in different sets of experiments. It does not produce, however, absolutely similar numbers of root-hair infections in each set of experiments, as discussed

previously (Niwa *et al*., 2007). For instance, the numbers of root-hair infections shown in Fig. 4 differed between the first and second experiments, although the percentages of spores without nucleus were similar. It is likely that the infection process is more sensitive to environmental factors (e.g. soil moisture and host nutrient status, which may affect root-hair development) than the germination process, thus the number of root-hair infections might be less reproducible than percentage germination. Replication of independent experiments, however, significantly improved the reliability of results obtained by the culture system.

In the present study, a reliable germination assay system under soil culture conditions in conjunction with nuclear/cell-wall double staining, was established. It was revealed that suppression of clubroot disease under neutral soil pH was caused by the inhibition of spore germination in the rhizosphere, although the suppressive effects of neutralization and calcium remain to be differentiated. Further investigations on the molecular aspects of spore germination would make a significant contribution to the understanding of the occurrence of clubroot disease.

Acknowledgements

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Figure captions

Figure 1 Resting spores of *Plasmodiophora brassicae* stained with 4'-6-diamidino-2-phenylindole-dihydrochloride. Spores with (arrowheads) or without a nucleus (arrows) were observed under UV-excitation. Scale bar, 10 µm.
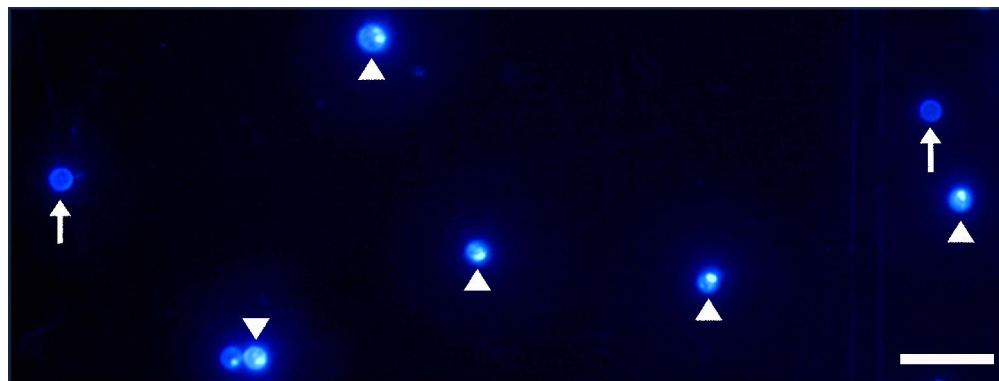
Figure 2 Effect of host plants on the mean percentage of *Plasmodiophora brassicae* spores without a nucleus stained with 4'-6-diamidino-2-phenylindole-dihydrochloride before and after incubation in glass-beads solution culture. The experiment was conducted twice (1st and 2nd) using different lots of inoculum. Vertical bars indicate standard errors ($n = 3$). Columns with different letters are significantly different (LSD test, $P<0.05$).

Figure 3 Resting spores of *Plasmodiophora brassicae* stained with Fluorescent Brightener 28 (UV-excitation) and SYTO 82 orange fluorescent nucleic-acid stain (G-excitation). The two photographs were taken from the same field under different excitations. Spores with (arrowheads) or without a nucleus (arrows) were discriminated under G-excitation. Scale bar, 10 µm.

Figure 4 Effect of host plants on the mean percentage of *Plasmodiophora brassicae* spores without a nucleus as assessed by the double staining method. Spores were

incubated for 7 days with *Brassica rapa* var. *perviridis* seedlings in a disease-conducive soil, then collected from the rhizosphere and non-rhizosphere soils and stained with Fluorescent Brightener 28 and SYTO 82 orange fluorescent nucleic-acid stain. The number of root-hair infections of *P. brassicae* was assessed after staining with aniline blue/phenol solution. The experiment was conducted twice (1st and 2nd) using different lots of inoculum. Vertical bars indicate standard errors ($n = 3$). Columns with different letters are significantly different (LSD test, $P<0.05$).

Figure 5 Effect of a) food factory sludge compost (FSC) and b) $CaCO_3$ on the mean percentage of *Plasmodiophora brassicae* spores without a nucleus, as assessed by the double-staining method. Spores were incubated for 7 days with *Brassica rapa* var. *perviridis* seedlings in the presence or absence of (a) 25 g FSC kg^{-1} soil or (b) 2.5 g $CaCO_3$ kg^{-1} soil, collected from the rhizosphere and non-rhizosphere soils and stained with Fluorescent Brightener 28 and SYTO 82 orange fluorescent nucleic-acid stain. The number of root-hair infections of *P. brassicae* was assessed after staining with aniline blue/phenol solution. Soil pH was measured after incubation. Vertical bars indicate standard errors ($n = 3$). Columns with different letters are significantly different (LSD test, $P<0.05$).

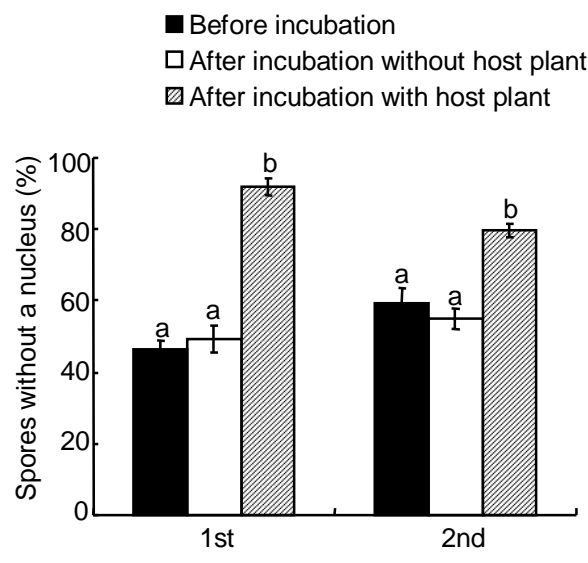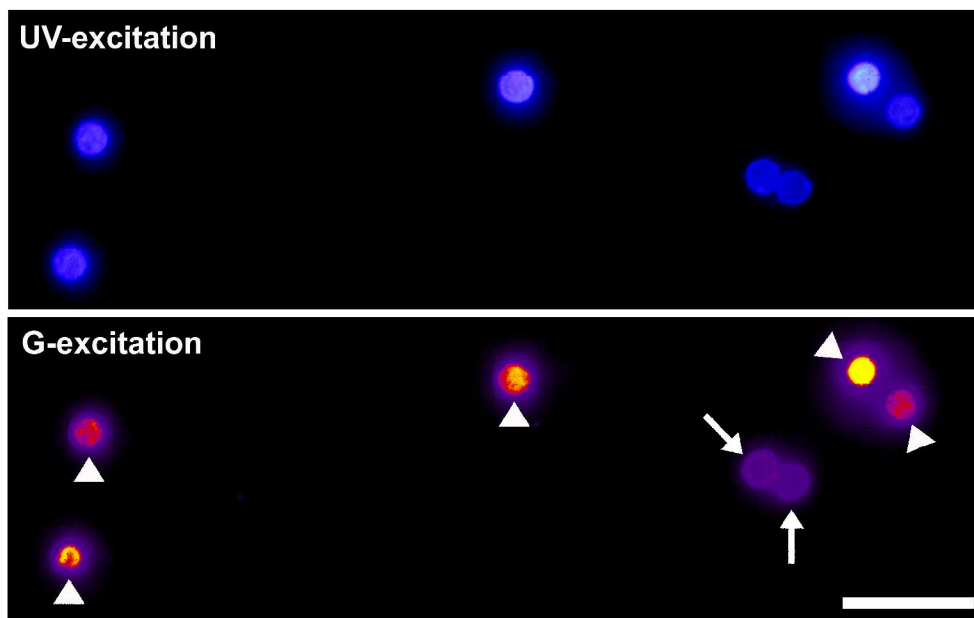**Figure 1**
308x116mm (300 x 300 DPI)

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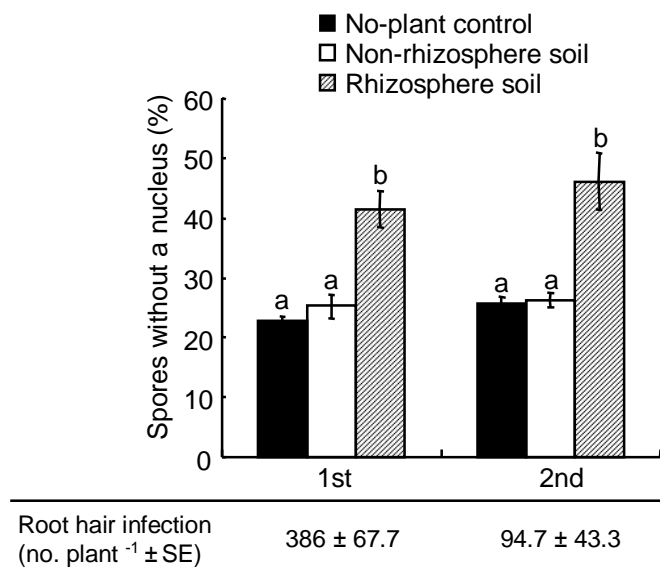51
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Figure 2

Niwa *et al*.
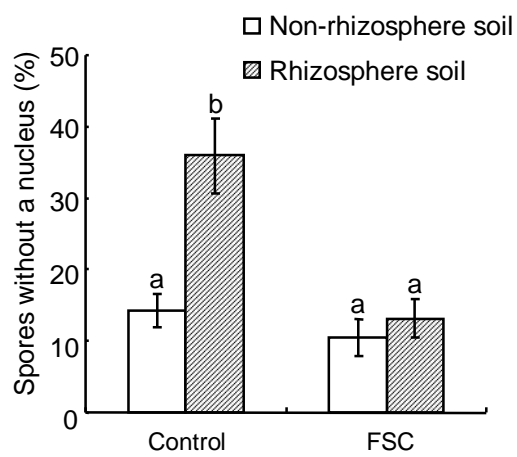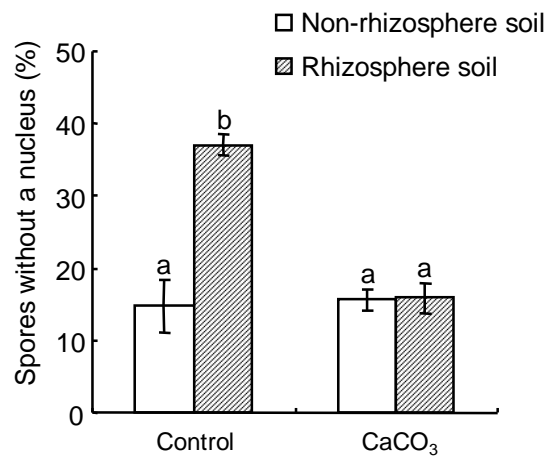
Figure 3
270x170mm (300 x 300 DPI)

■ No-plant control		
□ Non-rhizosphere soil		
▨ Rhizosphere soil		

Root hair infection (no. plant $^{-1}$ ± SE)	386 ± 67.7	94.7 ± 43.3

Figure 4
Niwa *et al*.

	Control	FSC
Soil pH	6.0	6.9
Root hair infection (no. plant $^{-1}$ ± SE)	233 ± 35 a	18.5 ± 6.6 b

Figure 5
Niwa *et al.*

	Control	CaCO$_3$
Soil pH	6.2	7.1
Root hair infection (no. plant $^{-1}$ ± SE)	298.6 ± 102.8 a	13.5 ± 5.8 b

Figure 6
Niwa *et al.*