土壌中におけるアブラナ科野菜根こぶ病菌休眠胞子の定量法

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An Improved Method for Estimating the Number of Resting Spores of *Plasmodiophora brassicae* in Soil

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Abstract

A method for the direct estimation of the number of resting spores of *Plasmodiophora brassicae* in soil by using a fluorescence microscope is described. After being mixed with tween 80 solution, the soil sample was filtered through sieves to remove coarse minerals and the filtrate was centrifuged. The pellet suspended in distilled water was mixed with a calcofluor white MR2 solution. The mixture solution was examined under a fluorescence microscope and the number of spores was counted. It was very easy to identify spores showing strong fluorescence. This method could be applied to infested soils containing 10^4 spores/g and slightly less, and also adopted for estimating the number of spores in different soil groups. This improved method has advantages due to the simplicity and shortening of the procedure, the easy enumeration of spores under a microscope and the high recovery efficiency of spores, as compared with the previous methods, and may be of value for practical use. Naturally infested soils in six fields were assessed for the contamination with spore concentrations of up to 10^5/g.

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Key words: *Plasmodiophora brassicae*, resting spores, estimation method.

Introduction

Clubroot of crucifers caused by *Plasmodiophora brassicae* is one of the major soil-borne diseases. It is important to determine the degree of infestation with *P. brassicae* in a given area of land.

To assess the inoculum density in field soil, some methods have been employed. Gall development observed on host plants grown in the test soil was not always associated with disease development in the field[5,8]. Observation of root hair infection on test plants is convenient as the time required for the assessment is short, as compared with the former method. However, it has been reported that there was no linear relation between root hair infection and clubroot development[6,7,10]. On the other hand, it is considered that the number of resting spores is closely related to the inoculum density in field soil. Fedotova[4] reported a method for counting the number of resting spores in soil suspensions under an optical microscope. However, this method is not generally used because it is difficult to identify resting spores in a soil suspension due to the presence of a large number of soil particles.

Buczacki and Ockendon[3] reported a method for the extraction of resting spores from

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soil by using a sucrose solution. Furthermore, Naiki and Kitazawa[11] improved the method of Buczacki and Ockendon[3] and obtained a high recovery of spores from artificially infested soil. However, these methods can not be extensively used because the procedures for the extraction of resting spores require a long time (4 days or more) as well as an experience or the availability of specialized equipment (an image analyser) for the identification of the spores.

Fluorochromes have been used to observe microorganisms in soil by some workers[1,2,12]. Miyata[9] observed the resting spores of *P. brassicae* in soil under a fluorescence microscope. These works suggested that fluorochromes may be suitable for the identification of the resting spores of *P. brassicae* in infested soil. The present paper describes an improved method for estimating the number of resting spores in soil by using a fluorescence microscope.

Materials and Methods

Artificially infested soils. Suspension of resting spores was obtained by grating frozen galls of infected plants and then centrifuging them according to the method of Horiuchi and Hori[6]. Artificially infested soils were prepared by adding the suspension of resting spores to the sterilized soils, in adjusting the number of spores per one gram of dry soil.

Observation and enumeration of spores. Resting spores in the soil samples were observed by using a reflected light fluorescence microscope (Olympus New Vanox AH2-RFL). The number of resting spores was counted by using a haemacytometer. The number of resting spores on 64 divisions (one division : 0.2×0.2 mm) in a haemocytometer was repeatedly counted thirty two times for the soils artificially infested with a low level of inoculum and naturally infested soil, and sixteen times for other soil samples. Spore concentration in soil was expressed by the number of spores per one gram of dry soil which was air-dried in an oven at 120 C for 3 days.

Selection of fluorochromes. The seven fluorescent substances tested were as follows, calcofluor white MR2 (0.1% in aqueous solution), acriflavine (0.01% in aqueous solution), acridine orange (0.01% in aqueous solution), eosin Y (0.1% in aqueous solution), aniline blue (0.1% in aqueous solution), fluorescein diacetate (20 μg/ml in phosphate buffer solution, 1/15 M, pH 7.5) and ethidium bromide (50 μg/ml in phosphate buffer solution, 1/15 M, pH 7.5). The substances were mixed (1 : 1 v/v) with artificially infested soil (an ando soil) containing a concentration of 1.0×10^7 spores/g dry soil. Then, the mixture solution were observed under a fluorescence microscope.

Method for extraction of spores from soil. At first, resting spores were extracted from infested soil according to a slightly modified version of the procedure improved by Naiki and Kitazawa[11], as shown in Fig. 1. Samples of 25 g of test soil (an ando soil) containing a concentration of 1.0×10^7 spores/g dry soil were mixed with detergents, *i. e.* 40 ml of 2% calgon (100% sodium hexametaphosphate) or 0.05% Tween 80 (polyoxyethylene sorbitan monooleate). Mixture solution was shaken for 2-3 hr on a wrist-action flaskshaker. Coarse organic matter and grit were removed by filtering the

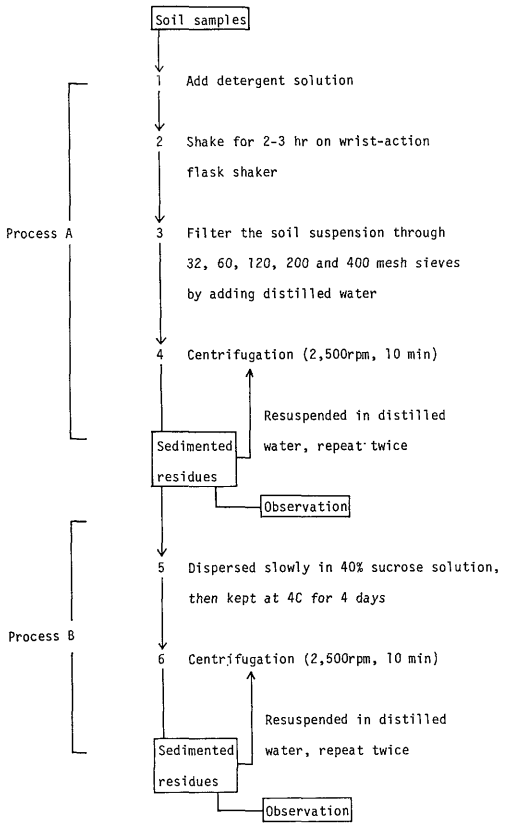

Fig. 1. Method for estimating resting spores of *Plasmodiophora brassicae* from infested soil, which is a slight modification of the procedure improved by Naiki and Kitazawa[11].

mixture solution through sieves of 32, 60, 120, 200 and 400 meshes by adding distilled water. The filtrate was centrifuged for 10 min at 2,500 rpm, the supernatant was discarded and the pellet was suspended in distilled water. This centrifugation procedure was repeated twice. A small amount of the soil suspension was used as sample for microscopic examination. The above mentioned procedure is designated as "A" process in Fig. 1, and the following procedure as "B" process in Fig. 1. The pellet was slowly dispersed in a 40% sucrose solution and the suspension was poured into a glass beaker. Beakers containing the soil extract in sucrose were placed in a refrigerator at 4 C for 4 days. After 4 days, the supernatant was carefully decanted and diluted with an equal volume of distilled water and then centrifuged for 10 min at 2,500 rpm. The pellet was suspended in distilled water. The suspension was centrifuged for 10 min at 2,500 rpm again. The pellet was resuspended in distilled water for microscopic examination. Soil suspensions used for microscopic examination were mixed with an equal volume of 0.1% calcofluor white MR2 solution, and then observed under a fluorescence microscope. In subsequent examinations, soil suspensions for fluorescence microscopy were prepared by the same method.

Detergents and amount of soil sample. Ten grams of artificially infested soil (an ando soil, spore concentration 1.0×10^7/g dry soil) were used as samples of test soils. Test soils were mixed with 20 ml of 2% calgon, 0.05% tween 80, 0.05% Tween

20 (polyoxyethylene sorbitan monolaurate) or distilled water, respectively. After the completion of the "A" process shown in Fig. 1, the recovery of resting spores from the test soils was evaluated for each detergent and distilled water.

Recovery of spores from different soil groups. In the recovery test of resting spores from the soil samples, five soil groups, a gray lowland soil (paddy soil), a red soil, a yellow soil (sandy loam), a yellow soil (clay loam) and an ando soil were employed. Each 10 g of these soil samples artificially infested with 1.0×10^7 spores/g dry soil was mixed with 20 ml of 0.05% tween 80. After the completion of the "A" process shown in Fig. 1, the number of spores in the soil suspension of the various soil groups was counted.

Recovery of spores from soils infested with low spore concentrations. Samples of 10 g of artificially infested soils (an ando soil) containing 1.0×10^6, 10^5, 10^4 and 10^3 resting spores/g dry soil respectively, were used as soil samples. Test soils were mixed with 20 ml of 0.05% Tween 80. After the completion of the "A" process shown in Fig. 1, the numbers of spores in the soil suspension were counted for each soil with different spore concentrations.

Estimation of numbers of spores in naturally infested soils. Concentrations of resting spores in the naturally infested soils of six different fields were estimated. A 10 g sample each of these soils was mixed with 20 ml of 0.05% tween 80. After the completion of the "A" process shown in Fig. 1, the number of spores in each soil suspension was counted.

Results

Fluorescence microscopy of resting spores

Seven fluorochrome solutions mixed with infested soils were observed with a variety of combinations of exciter and barrier filters. Among the fluorochromes tested, calcofluor white MR2 (0.1% in aqueous solution) induced the strongest fluorescence on resting spores. The optimum filter combination for this brightener-induced fluorescence was the exciter filter BP405 (transmits ca. 395-415 nm with 405 nm peak) and barrier filter Y455 (transmits above 445 nm). Resting spores showing strong bluish yellow fluorescence could be easily distinguished from soil particles or other microorganisms due to their globular shape and their size (3-5 μm in diameter). This brightener-induced fluorescence did not fade readily with prolonged viewing. However, the spores were frequently shaded by large soil particles and for the enumeration of spores it was necessary to remove large soil particles from the soil suspension.

Removal of soil particles

An efficient method for removing soil particles from the infested soil was examined. Soil suspensions obtained by the two steps in the procedure shown in Fig. 1, *i. e.* after "A" process (pre-sucrose treatment) and after "B" process (post-sucrose treatment), were observed under a fluorescence microscope. Soil suspension observed prior to the sucrose treatment still contained large numbers of small soil particles and other microorganisms, but the spores could be easily identified and enumerated (Fig. 2). The ratio

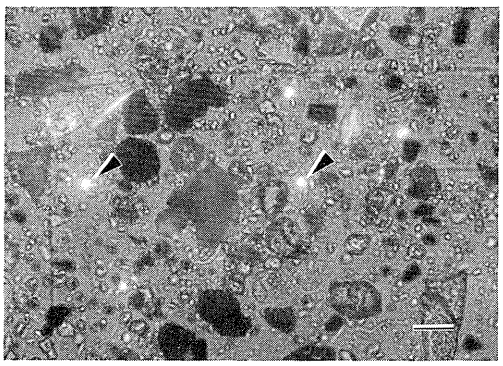

Fig. 2. Fluorescence micrograph of resting spores of *Plasmodiophora brassicae* in soil suspension. Bar represents 20μm.

of the number of spores counted to the number of spores originally added was higher for the pre- than post-sucrose treatment when both calgon and tween 80 were used as detergents (Table 1). Recovery efficiency of spores when tween 80 was used was significantly reduced by the sucrose treatment, as compared with calgon use. The "B" process was not necessary for spore enumeration. There were no differences in recovery efficiencies of spores prior to the sucrose treatment between calgon and Tween 80. However, the brightener-induced fluorescence on spores was weaker in the case of calgon. In this experiment, a 25 g sample of dry soil was too large to enable to remove completely soil particles.

Selection of detergents and amount of soil samples

To determine which detergent is most suitable for inducing an intense and characteristic fluorescence on spores, recoveries of spores from 10 g samples were examined when calgon, tween 80 and tween 20 were used as detergents. Recovery efficiencies for the three detergents and distilled water were high and scarcely different (Table 2). The fluorescence on spores with the use of tween 80, tween 20 and distilled water was strong enough to allow for spore enumeration unlike in the case of calgon. Therefore, tween 80 and tween 20 were recommended as detergents, and also distilled water could be used instead of the detergents.

Table 1. Recovery of resting spores of *Plasmodiophora brassicae* from artificially infested soil during the various steps of the procedure[a)]

Detergents used		Time when resting spores are counted	
		Prior to sucrose treatment	After sucrose treatment
Calgon	1	98.1[b)]	96.6
	2	97.5	94.2
Tween 80	1	100.0	40.4
	2	98.0	40.2

a) Procedure is shown in Fig. 1.
Soil sample: 25g, ando soil, spore concentration 1.0×10^7/g dry soil.

b) Percentage of recovery of spores (%):
Number of spores counted × 100/number of spores originally added.

Table 2. Effect of detergent on recovery of resting spores of *Plasmodiophora brassicae* from artificially infested soil and intensity of fluorescence on spores[a)]

Detergents	Recovery efficiency of resting spores (%)[b)]	Intensity of fluorescence on spores
Calgon	97.2 ± 3.2[c)]	+[d)]
Tween 80	103.6 ± 6.4	++
Tween 20	104.7 ± 4.9	++
Distilled water	102.1 ± 4.0	++

a) Soil sample: 10g, ando soil, spore concentration 1.0×10^7/g dry soil.
b) Number of spores recovered × 100/number of spores originally added.
c) Mean and standard deviation in three experiments.
d) Degree of intensity of fluorescence estimated visually: ++ = strong, + = weak.

The 10 g soil sample used in this experiment allowed for a high efficiency of recovery of spores. Therefore, the use of a 10 g sample instead of 25 g[3,11] was recommended as the optimum amount of soil sample.

Application of the method to different soil groups

Among the five soil groups tested, the gray lowland soil (paddy soil), red soil, yellow soil (sandy loam) and yellow soil (clay loam) gave high efficiencies of recovery of spores (Table 3). The recovery efficiency of spores from an ando soil was slightly low, as compared with that from the other soil groups.

Table 3. Recovery of resting spores from several soil groups artificially infested with *Plasmodiophora brassicae*

Soil groups tested[a)]	Recovery efficiency of resting spores (%)[b)]	
	Exp. 1	Exp. 2
Gray lowland soils (Paddy soil)	100.1	103.8
Red soils	103.1	101.6
Yellow soils (Sandy loam)	90.6	100.8
Yellow soils (Clay loam)	105.3	102.4
Ando soils	96.2	93.0

a) Soil sample: 10g, spore concentration 1.0×10^7/g dry soil.
b) Number of spores recovered × 100 / number of spores originally added.

Table 4. Recovery of resting spores of *Plasmodiophora brassicae* from soil artificially infested with spores of various concentrations

Number of resting spores originally added[a)] (1.0 × /g dry soil)	Number of resting spores recovered (/g dry soil)	
	Exp. 1	Exp. 2
10^6	1.1×10^6	1.0×10^6
10^5	1.2×10^5	0.8×10^5
10^4	5.5×10^4	1.1×10^4
10^3	6.1×10^3	$<6.1 \times 10^3$

a) Soil sample: 10g, ando soil.

Table 5. Concentration of resting spores of *Plasmodiophora brassicae* estimated in naturally infested field soils

Fields examined	Vegetables cultivated	Disease severity	Soil pH	Number of resting spores (/g dry soil)
Shin-asahi, Shiga (I)	Turnip	Severe	6.7	3.3×10^5
do (II)	Turnip	Mild	8.0	2.1×10^5
Shiga Pref. Agric. Exp. Stn.	Turnip	Severe	6.7	1.2×10^5
Chugoku Natl. Agric. Exp. Stn.	Chinese cabbage	do	7.0	2.3×10^5
Kamigamo, Kyoto	Turnip	do	6.5	1.0×10^6
Kameoka, Kyoto	Turnip	do	7.4	1.6×10^6

In this and former experiments, 10 g samples of artificially infested soil containing 1.0×10^7 spores/g dry soil were repeatedly employed for the recovery of spores. Through these experiments, the average efficiency of recovery of spores was $100.9 \pm 5.4\%$ for an ando soil and $100.9 \pm 5.0\%$ for all the soil groups including the ando soil.

Application of the method for soils with low spore concentrations

The numbers of spores counted in the suspension from soils containing 10^6, 10^5 spores/g dry soil gave good indications of the number originally added to the soil (Table 4). On the other hand, the relationship between spore contents of soil sample and the counts obtained for soils containing 10^4 spores/g was not significant, as compared with that for soils containing 10^6 or 10^5 spores/g. However, the numbers of spores recovered from soils containing 10^4 spores/g were not remarkably different from the numbers of spores originally added to the soil samples. In this experiment, the recovery of spores from soil containing 10^3 spores/g could not be theoretically expected due to the limitation in the number of microscope fields counted. To estimate the number of spores in soil inoculated with 10^3 spores/g, counts of several hundreds of microscope fields were needed for the calculation. However, the numbers of spores counted in suspension from soils containing 10^3 spores/g were less than 10^4 spores/g.

Estimation of spore load in naturally infested soils

All the naturally infested soils from the six fields examined, showed spore loads of up to 10^5/g (Table 5). Two fields at Shin-asahi in Shiga Prefecture showed a difference in disease development of clubroot, although they appeared to contain almost the same spore load. However, the soil pH value of these fields was significantly different, and the soil pH of the field where severe symptoms of clubroot developed was lower than that of the other fields.

Discussion

The methods hitherto employed for the direct estimation of the number of resting spores of *P. brassicae* in soil made it difficult to identify the spores due to the presence of large numbers of soil particles. However, the difficulty was solved by using a fluo-

rescence microscope after staining infested soils with a fluorochrome, calcofluor white MR2.

In the methods for the estimation of resting spores in soil described by Buczacki and Ockendon[3] and Naiki and Kitazawa[11], the spores were counted by using differential interference contrast optics or a phase contrast microscope. However, these methods required the treatment with a sucrose solution to extract spores after removing coarse mineral particles from soil. The treatment with a sucrose solution required 4 days and more to extract spores. On the other hand, the use of a fluorescence microscope enables to count spores in a soil suspension containing fine mineral particles, and does not require the treatment with a sucrose solution. Therefore, the method described in this paper was improved as the use of a fluorescence microscope enabled to shorten the procedure. Buczacki and Ockendon[3] reported that they obtained a recovery efficiency of 100% of resting spores from artificially infested soil. Then, Yoshikawa *et al.*[13] and Naiki and Kitazawa[11] reexamined the method described by Buczacki and Ockendon[3], but could recover only 59-69% and 84.0±13.0% of spores, respectively. Naiki and Kitazawa[11] which could obtain a recovery of 95.0±2.0% of spores from a 25 g sample of artificially infested soil containing spores at a concentration of 10^8/g dry soil by improving of the Buczacki and Ockendon's method, also reported that some spores were lost during the sucrose solution treatment. The improved method described in this paper enabled to obtain a recovery of 100.9±5.0% of spores from a 10 g sample of artificially infested soil containing spores at a concentration of 10^7/g dry soil. This high recovery efficiency suggests that the loss of spores in the sucrose solution could be prevented. This high recovery efficiency was achieved from a small amount (10 g) of soil sample and without requiring ultrasonic treatment of the soil suspension. Therefore, this improved method has the advantage of allowing for a high efficiency of recovery of spores while the amount of soil sample is small and the procedure is simple, as compared with the previous methods[3,11].

There are various groups of soil in fields. By this improved method it was possible to obtain a satisfactory recovery of spores from the five different soil groups. It is thus suggested that this method may be applicable to various soils.

Recovery of resting spores from soil artificially infested with a low spore concentration of 10^6-10^3/g dry soil was achieved by using the improved method. Estimations of numbers of spores in soils containing 10^6 or 10^5 spores/g were remarkably accurate. In soil containing 10^4 spores/g, the estimation of the spore concentration was less accurate, as compared with that in soils containing 10^6 or 10^5 spores/g, because the number of spores observed in the soil suspension was low. However, the enumeration of spores under a fluorescence microscope did not require much time. Therefore, since the number of microscope fields where counts are performed can be increased, it is considered that this improved method can be satisfactorily applied to soils with a low level of infestion that contain 10^4 spores/g. Also this method could be applied to soils containing approximately 10^3 spores/g by increasing the number of microscope fields for the counts. It has been reported that the development of clubroot increased with the rise in the spore load of soil within the range of 10^1 to 10^7 spores/g soil[6,11]. However, Horiuchi

and Hori[6] observed a severe incidence of the disease for a spore number of $3\text{-}4\times10^5$/g soil and higher. In this experiment, the naturally infested soils where severe symptoms of clubroot developed contained more than 10^5 spores/g dry soil. It is well-known that the environmental conditions influence the effect of spore load on clubroot development. However, the current method can be applied to soils containing 10^4 spores/g and slightly less. It thus appears that this method may be useful for estimating spore loads in field soil.

The application of the method requires a fluorescence microscope and a simple centrifuge but only 10 g of soil samples. By sending soil samples to laboratories where such equipments are available, the method could be applied to farmers' fields.

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和 文 摘 要

高橋賢司・山口武夫：土壌中におけるアブラナ科野菜根こぶ病菌休眠胞子の定量法

土壌中における根こぶ病菌（*Plasmodiophora brassicae*）休眠胞子の定量は，これまで長時間の操作と休眠胞子の判別に熟練を必要とした。そこで螢光顕微鏡を利用した簡易な定量法について検討した。汚染土 10 g に tween 80（0.05%水溶液）を混合し，2～3時間撹拌した。その土壌懸濁液を32, 60, 120, 200および400メッシュの篩で順次沪過した後，沪液を遠心分離（2,500 rpm, 10分間）した。沈澱部を蒸留水に懸濁した後，再度遠心分離して同様の操作を行い被検液を調製した。被検液を calcofluor white MR2（0.1%水溶液）と等量混合後，落射螢光顕微鏡で観察した。休眠胞子は螢光発色し，微小土壌粒子と明瞭に判別され，休眠胞子数の定量が可能であった。本定量法は，休眠胞子濃度 10^5個/g・乾土以上の高濃度汚染土で高い検出精度が得られ，10^7 個の汚染土からの休眠胞子の回収率は100.9±5.0%であった。10^4 個以下の低濃度汚染土では検出精度が高濃度の場合ほど高くなかったが，10^3 個付近まで定量は可能であった。本定量法は，操作と休眠胞子観察の簡易性，検出精度の点において従来の定量法より優れていると考える。また本定量法は，異なる5種の土壌に対し適用できた。本定量法を用いて根こぶ病発生圃場における休眠胞子密度を調べた結果，調査した6圃場全てで 10^5個/g・乾土以上の休眠胞子が検出された。