Influences of Some Environmental Factors on the Viability of Resting Spores of *Plasmodiophora brassicae* Wor. Incubated in Sterile Soil

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Abstract

Influences of environmental factors such as temperature, soil moisture and soil pH on the viability of resting spores of *Plasmodiophora brassicae* incubated in sterile soil were examined by using the direct method based on the fluorescent staining reaction in spores. When resting spores were incubated in acid soil, the frequency of nonviable spores increased readily and profusely after incubation and reached approximately 70 to 80% after a 30-day incubation. The rate of the increase was not appreciably affected by temperatures between 15 and 30°C and by soil moisture contents between 35 and 100% during the 30-day incubation, though affected slightly by low temperature and moisture content. The temperatures and soil moistures in the range tested were not so important factor as affecting the viability of resting spores. On the other hand, soil pH distinctly affected spore viability. A remarkable increase in the nonviable spore frequency was found in acid soils with pH values of approximately 4.5-6.5. In alkali soils the rate of increase was conspicuously low in the beginning of incubation period as compared with those in acid soils, but then the nonviable spore frequency increased continuously at an almost constant rate during a 7-month incubation. The soil pH is one of important factors affecting the viability of resting spores, but may not be the determinate factor in their survival due to lack of ability to suppress independently the decline of the viability during a long period of time.

(Received March 30, 1994)

Key words : *Plasmodiophora brassicae*, resting spores, viability, survival.

INTRODUCTION

Club root, caused by the soilborne fungus *Plasmodiophora brassicae* Wor., is an important disease of cruciferous vegetables in many regions of the world. The fungus produces large numbers of resting spores inside gall tissues on infected hosts. Resting spores are able to act as the sole inoculum source in soils. Resting spores germinate in the presence of a suitable plant and release primary zoospores, which penetrate into the root hairs. Resting spores, which germinate in the absence of a suitable plant, on the other hand, lose their infective ability since the primary zoospores can not survive in soils over a long period of time due to lack of saprophytic ability. However, since it is well known that resting spores can remain viable for many years in soils^{4,8)}, it is likely that resting spore germination must be suppressed in soils without a suitable plant. Environmental factors such as temperature, soil moisture and soil pH severely affect infection and disease development^{2,6,10,12,13,23)}. Resting spore germination in suspension is also influenced by factors such as temperature, pH, and the presence of host roots^{3,11,14,15,21)}. On the other hand, the factors affecting the viability of resting spores in soils have not been elucidated sufficiently, although they have been studied for a long duration, as reviewed by Colhoun⁴⁾ and Karling⁸⁾, for the reason that the prolonged survival of resting spores in fields is one of the chief obstacles to

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eradication of this disease.

Almost all tests of resting spore viability in soils have been conducted by using indirect methods because of the difficulty to identify the viable or germinated spores in soils. Indirect methods based on root hair infection or disease incidence are not reliable for tests of spore viability due to various influences on the processes of fungal infection and clubroot development^{1,4,8,16)}. The direct method based on a observation of difference in the fluorescent staining reaction in spores by fluorescence microscopy has been developed for assessing the viability of resting spores of *P. brassicae* in soil by the authors²⁰⁾. This method could distinguish nonviable spores as red spores showing red fluorescence in spores, from viable spores. Red spores involved nonviable inactive and germinated spores because the germinated spores recognized by Nomarski differential interference contrast microscopy were identified as red spores by fluorescence microscopy²¹⁾.

In this study, resting spores of *P. brassicae* are incubated in sterile soil without host roots to omit the influences of biological factors, and the influences of some environmental factors on their viability are examined in detail by using the direct method.

MATERIALS AND METHODS

Infested soil. A spore suspension from frozen clubroot galls was prepared according to the method described by Horiuchi and Hori⁶⁾ and diluted with sterile deionized water to adjust the spore concentration by using a haemocytometer. The soils used in the experiments were collected from an uncultivated area on Hiruzen Hill in Okayama Prefecture, which were an ando soil of pH 4.6. The soil was screened through a 1 mm mesh sieve, then autoclaved for 1 hr at 120°C. Artificially infested soils were prepared at the concentration of 1.0×10^7 spores per gram dry soil by the addition of spore suspensions to the autoclave-sterilized soils.

Soil moisture content. Moisture content in the infested soils calculated on an oven-dry soil basis (105°C, 24 hr) was adjusted by the amount of spore suspension added. The soil moisture contents were corrected to the initial values by the addition of sterile deionized water at the time when the tests of spore viability were conducted during the incubation.

Soil pH. Adjustment of soil pH was carried out by the addition of various types of calcium salts into the infested soils. The soil pH values were examined immediately after the addition of salts and at the time when the tests of spore viability were conducted during the incubation. The soil suspension prepared by mixing well 20 g of soil in 50 ml deionized water, was kept for more than 1 hour, then the pH was determined with a pH meter.

Incubation. Ten grams of infested soils were tightly sealed in a polyethylene bag and incubated in a controlled-temperature chamber. The effect of temperature on spore viability was examined at 15, 20, 25 or 30°C by using soils with a 55% moisture content. The effect of soil moisture on spore viability was examined in soils with 35, 55, 75 or 100% moisture contents that were incubated at 25°C. The effect of soil pH on spore viability was examined in the soils with a 55% moisture content that were incubated at 25°C.

Spore viability. Viability of resting spores was examined by the differential fluorescent staining method described previously^{19,20)}. The staining solution consisted of a mixture of equal volumes of ethidium bromide (50 µg/ml) and calcofluor white M2R (100 µg/ml) aqueous solutions. The samples for fluorescence microscopy were prepared by suspending the infested soil in the staining solution. When the samples were observed by fluorescence microscopy, the spores could be readily identified in soil by the presence of a strong blue fluorescence on the wall layer, and classified into two groups, *i.e.*, viable spores showing nonfluorescence (blue spores) and nonviable spores showing red fluorescence in their inner aspect (red spores). The percentage of red spores corresponding to the nonviable spores was determined for each sample. At least 300 spores were counted in each sample.

RESULTS

Influences of temperature and soil moisture on spore viability

The effect of temperature on spore viability in acid soils of pH 4.6 with a 55% moisture content is shown in Fig. 1. The percentages of red spores continuously increased after incubation at all temperatures, particularly at high temperatures. A significant difference in the red spore frequencies among the temperatures was only observed in the range between 15 and 30°C after a 10-day incubation. Red spore frequencies at low temperatures gradually increased after a 10-day incubation. The percentage of red spores for each temperature reached approximately 70 to 80% after a 30-day incubation, and did not show any appreciable differences among the temperatures. The effect of soil moisture on spore viability incubated at 25°C in the acid soils is shown in Fig. 2. The percentages of red spores in soils with a high moisture content rapidly increased after incubation. A significant difference in the red spore frequencies

Fig. 1. Effect of temperature on the viability of resting spores of *Plasmodiophora brassicae* incubated in sterile soil. Nonviable spores are shown as red spores in figure. The infested soils with a pH of 4.7 and a 55% moisture content were incubated at 15, 20, 25 or 30°C for 30 days. Data are means of four replicated experiments.

Fig. 2. Effect of soil moisture content on the viability of resting spores of *Plasmodiophora brassicae* incubated in sterile soil. Nonviable spores are shown as red spores in figure. The infested soils with a pH of 4.7 and moisture contents of 35, 55, 75 or 100% were incubated at 25°C for 30 days. Data are means of four replicated experiments.

Fig. 3. Effect of soil pH adjusted by the addition of calcium hydroxide on the viability of resting spores of *Plasmodiophora brassicae* incubated in sterile soil. Nonviable spores are shown as red spores in figure. The infested soils with a 55% moisture content and pH values adjusted by the addition of $\text{Ca}(\text{OH})_2$ at various concentrations or nonamended, were incubated at 25°C for 30 days. The percentages of red spores and soil pH values were examined 0, 15 and 30 days after incubation. Data are means of four replicated experiments. Bars indicate standard errors.

was detected in the range between 35% and other moisture contents only after a 20-day incubation. After a 30-day incubation, however, no differences in red spore frequencies were detected among the soils. Thus, the frequency of nonviable spores expressed as the percentage of red spores was not appreciably influenced by the temperature and soil moisture content in the range tested, though slightly influenced by low temperature and moisture content.

Influences of soil pH on spore viability

The infested soils with a 55% moisture content and pH values adjusted by the addition of calcium hydroxide at concentrations of 0.625, 1.25, 2.5 or 3.75 g per 100 g dry soil weight, were incubated at 25°C for 30 days. Figure 3 shows the soil pH values and the percentages of red spores at 0, 15 and 30 days after incubation. The soil pH, which increased with the increase of the additional amount of $\text{Ca}(\text{OH})_2$, slightly decreased by incubation. On the other hand, although the percentages of red spores increased considerably by incubation in all the soils, the rate of increase was lower in the soils with higher pH values. The infested soils with a 55% moisture content and pH values adjusted by the addition of various types of calcium salts such as CaCl_2 , CaNO_3 , CaSO_4 , CaCO_3 and $\text{Ca}(\text{OH})_2$ at 2.5 g per 100 g dry soil weight, were incubated at 25°C for 30 days. Soil pH values and the percentages of red spores at 0, 15 and 30 days after incubation are shown in Fig. 4. The soil pH values decreased by the addition of CaCl_2 , CaNO_3 and CaSO_4 , but increased by the addition of CaCO_3 and $\text{Ca}(\text{OH})_2$, as compared with the values in the nonamended control soil. The changes in the soil pH values with incubation detected in the soils to which these calcium salts had been added, were not appreciable. On the other hand, the percentages of red spores increased with incubation in all the soils. However, the rate of increase was lower in the soils with higher pH values. The pH affecting the viability of spores in soil was examined based on the percentage increase of red spores for 15 days when the infested soils with a 55% moisture content and pH values adjusted by the addition of calcium salts at various application were incubated at 25°C. The results are shown in Fig. 5. The maximum value of the percentage increases of red spores corresponded to a pH value of approximately 5.5. The percentage increases of red spores were markedly reduced in acid soils with a lower pH value than approximately 4.5 and in alkali soils with a higher pH value than approximately 6.5. The pH, which could remarkably affect the viability of spores in soil, was the range

Fig. 4. Effect of soil pH adjusted by the addition of various calcium salts on the viability of resting spores of *Plasmodiophora brassicae* incubated in sterile soil. Nonviable spores are shown as red spores in figure. The infested soils with a 55% moisture content and pH values adjusted by the addition of various calcium salts at 2.5 g per 100 g dry soil weight or nonamended, were incubated at 25°C for 30 days. The percentages of red spores and soil pH values were examined 0, 15 and 30 days after incubation. Data are means of four replicated experiments. Bars indicate standard errors.

Fig. 5. Optimum soil pH for the frequency increase of nonviable resting spores of *Plasmodiophora brassicae* incubated in sterile soil. Nonviable spores are shown as red spores in figure. The infested soils with a 55% moisture content and pH values adjusted by the addition of various types of salts at various concentrations or nonamended, were incubated at 25°C for 15 days. The percentage increases of red spores were calculated by the differences between the frequencies examined 0 and 15 days after incubation. Data are means of twice replicated experiments. 'A1' and 'A2' above column represent the range of optimum suspension pH for resting spore germination, as indicated by Narita and Nishiyama¹⁵⁾ and Naiki¹⁴⁾ respectively. 'B' above column represents the range of optimum soil pH for infection and disease development, as indicated by Colhoun⁴⁾, Karling⁸⁾ and Naiki¹⁴⁾.

Fig. 6. Viability of resting spores of *Plasmodiophora brassicae* incubated for a long period of time in sterile soil. Nonviable spores are shown as red spores in figure. The infested soils with a 55% moisture content and pH values adjusted by the addition of calcium hydroxide at various concentrations or nonamended, were incubated at 25°C for 7 months. Numerals in column represent soil pH values. The pH value of nonamended infested soil as control was 4.7 at the beginning of incubation. Data are means of four replicated experiments. Bars indicate standard errors.

from approximately 4.5 to 6.5.

The infested soils with a 55% moisture content and pH values adjusted by the addition of $\text{Ca}(\text{OH})_2$ were incubated for 7 months. The pH values and the percentage increases of red spores were examined at monthly intervals, respectively. The results are shown in Fig. 6. In acid soils the percentages of red spores increased rapidly and profusely after incubation, then were rising slightly but continuously with the time of incubation and reached above 95% after a 7-month incubation at the end of experiment. On the other hand, in alkali soils the percentages of red spores increased gradually through a incubation period. After a 7-month incubation the percentages of red spores in alkali soils reached comparatively high values of above 70%. The differences in the percentages of red spores between acid and alkali soils, therefore, were pronounced in the beginning of incubation period, but were not so conspicuous after a 7-month incubation. The initial value for each soil pH decreased with the time of incubation, particularly in the alkali soils in the range from 9.2 to 7.2, or from 8.2 to 6.6. after a 7-month incubation period. These falling pH values (7.2 and 6.6), however, were still higher than the optimum soil pH range (4.5–6.5) for the frequency increase of red spores.

DISCUSSION

When resting spores of *P. brassicae* were incubated in soils excluded influences of biological factors such as host roots or soil microorganisms, the frequency increase of nonviable spores expressed as red spores was observed. In acid soils the frequency increased readily and profusely up to approximately 70–80% for a 30-day incubation, and then reached high level above 95% after incubation of several months by a gradual increase. This aspect of decline in spore viability in acid soils, obtained by using a direct method, that was almost coincident with the results obtained by indirect methods based on root hair infection¹⁰. Macfarlane¹⁰ assumed that the viable resting spores, which were not deactivated in the beginning of incubation period, would be able to survive as dormant spores for long periods in soils unless they were affected by biological agents such as host roots. However, this study showed the frequency of nonviable spores continued to increase gradually in spite of the absence of host roots. The presence of host roots appeared unlike an essential factor in the survival of resting spores in sterile soils. Environmental factors such as temperatures, soil moistures and soil pH could affect the degree of clubroot infection and disease development^{2,5,6,10,12,13,16,23}. The present results, however, showed that the spore viability was not appreciably affected by the temperature (15–30°C) and soil moisture (35–100%)

during a 30-day incubation period, though was slightly affected by low temperature and moisture content. So far as the range tested in this experiment, temperature and soil moisture were not so important factor as affecting the viability and survival of resting spores in soil. On the other hand, soil pH could distinctly affect spore viability. The increases of nonviable spore frequencies were suppressed in alkali soils during a 30-day incubation period, as compared with those in acid soils. Soil pH must be one of important factors affecting the viability of resting spores in soil, as suggested by the experiments based on the indirect methods^{2,5,6,10,12,13,23}. However, the frequencies of nonviable spores increased with the time of incubation even in alkali soils, though were lower than those in acid soils even after a 7-month incubation. Thus, since the soil pH appeared to be insufficient in the ability to suppress independently the frequency increase of nonviable spores, it may not be an essential factor on the survival in soils extend over many years. In this study, the determinate factor for the survival of resting spores were not detected when incubated in sterile soils without host roots.

Red spores involved nonviable inactive and germinated spores¹⁹⁻²¹. In this study, it was indistinguishable whether the cause of the frequency increase of nonviable spores was spore inactivation or germination. Environmental conditions and antifungal substances are considered as factors deactivating resting spores in soils. Some reports indicate that high temperatures and drought can deactivate resting spores^{7,9,12,13,19,20,23}, but the highest temperature (30°C) tested in this experiment during incubation was not high enough to deactivate spores because the optimum temperature for resting spore germination in a suspension was in the range of 20-30°C^{3,14,15}. The present results demonstrated that the nonviable spore frequency in the soil with a 35% moisture content was less than that in soils with higher moisture contents. This indicates that a 35% of soil moisture content is not low enough to deactivate resting spores. Biological antifungal agents, an important factor deactivating spores, were excluded from this study because of the incubation in sterile soils. On the other hand, nonbiological antifungal substances may be produced newly by autoclave-sterilization. However, in gas-sterilized soil, like as in autoclave-sterilized soil, a larger increase in the nonviable spore frequency was found, as compared with that in non-sterile soil²². This implies that antifungal substances as a production of autoclave-sterilization are not the main causal agent to increase the nonviable spore frequency. Therefore, it is thought that the frequency increases of nonviable spores observed in this study are ascribed to mainly spore germination, not to inactivation.

These interpretations were supported by the present results that the increase of nonviable spore frequency was distinctly influenced by soil pH as if spore germination in suspension^{14,15}. The optimum range of soil pH for the frequency increase of nonviable spores in soil, which was approximately 4.5 to 6.5, coincided with that for infection and disease development (approximately 4.6-6.5)^{4,8,14}, though the range was wider when compared with that for spore germination in suspension (approximately 6.0-6.5)^{14,15}. The soil pH may be able to affect spore germination in soil, as well as infection and disease development, and play an important part in the primary stage of infection of *P. brassicae*. Resting spore germination in suspension is stimulated by the presence of host roots, or the exudates from and extract of host roots^{11,15,18,21}. Some studies also presumed the existence of factors stimulating resting spore germination in host root exudates^{11,18}. These studies indicated that the presence of host roots is an essential factor for resting spore germination. On the other hand, Narita and Nishiyama¹⁵ found that resting spore germination were stimulated not only by host roots but also by soil filtrate. Smith¹⁷ reported that resting spores could well germinate in non-sterile soil extract, though was not confirmed by Naiki¹⁴. These reports suggest that factors stimulating the germination of resting spore are existing in soil. The present results also showed that the nonviable spore frequency increased remarkably in sterile soil in spite of the absence of host roots, as supporting the reports above. Although resting spores can survive in fields extend over many years, however, factors suppressing the increase of nonviable spore frequency have not been detected yet. Analyses of factors affecting the viability and survival of resting spores in natural soil in fields will be required.

The author is grateful to Dr. H. Date, Okayama Agricultural Experiment Station for supplying the soil samples and wishes to thank Dr. H.W. Israel, Cornell University (present address: Agritech. Inc.) and Dr. K.

Abiko, Chugoku Agricultural Experiment Station for the critical reading of the manuscript.

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和 文 摘 要

高橋賢司：滅菌土壤中に培養したアブラナ科野菜根こぶ病菌休眠胞子の活性に及ぼす環境要因の影響

滅菌土壤中に培養したアブラナ科野菜根こぶ病菌 (*Plasmodiophora brassicae*) 休眠胞子の活性に及ぼす温度、土壌水分および土壌 pH の影響を調べた。酸性土壌中では不活性胞子の割合は、宿主植物根が不在でも培養直後から速やか

かつ顕著に増加し、培養30日後に約70～80%に達した。この培養直後の不活性胞子の割合の増加は、温度(15～30℃)と土壤水分(含量35～100%)にほとんど影響されなかったが、土壤pHには大きく影響され、pHが約4.5～6.5の酸性土壤で増加が大きかった。しかし、アルカリ性土壤中でも不活性胞子割合の増加は完全に抑制されず、7カ月間の培養中に徐々に割合が増加した。これらの結果から、土壤pHは休眠胞子の活性に大きく影響する要因であるが、休眠胞子の生存を単独で決定できる要因ではないことが示唆された。