A Simple Inoculation Method with a Single Resting Spore of *Plasmodiophora brassicae*

Koji KAGEYAMA*, Yoriko KAMIMURA** and Mitsuro HYAKUMACHI*

Abstract

Single inoculation method using a single resting spore of *Plasmodiophora brassicae* was developed. Single resting spore was obtained by placing a 0.5 μ l drop of spore suspension, which was adjusted to the concentration of 2000 spores per ml, on a piece of sterilized cover glass with a micropipette. The drop was checked under an inverted microscope to ensure that it exactly carried one spore alone. The cover glass carrying a single spore was placed on the surface of autoclaved soil in a pot. One- or two-day-old seedling of Chinese cabbage was inoculated by placing over the spore drop, covered with a small amount of soil, and grown in a growth chamber. The inoculation method could give an efficient infection. Pathogenicity of the single-spore isolates obtained was compared with their original populations to the host lines of European Clubroot Differential set, and was not always identical in those combinations.

(Received September 9, 1994; Accepted March 20, 1995)

Key words : crucifers, *Plasmodiophora brassicae*, single-resting-spore inoculation.

INTRODUCTION

Clubroot disease, caused by *Plasmodiophora brassicae* Wor., is one of the most important diseases of cruciferous plants and breeding for resistant cultivars is in progress. It is suggested, however, that a wild population of *P. brassicae* consists of some pathotypes even in a field plot or within a gall tissue^{3,4,13}. Accordingly, single spore isolation is essential for purification of pathotypes. The trial, however, has been difficult since *P. brassicae* cannot be artificially cultured, except for callus culture of infected host tissue with the pathogen⁴. Although isolates have been obtained by inoculating seedlings with a single resting spore^{1,6,8,10}, the techniques were laborious and often failed to give a high success rate.

This paper describes studies for an improved and simplified method for producing shingle-spore isolates, and differential pathogenicity of the isolates.

MATERIALS AND METHODS

Isolation of single resting spores. Clubroot galls were collected from Prefectures of Aichi and Ibaraki in Japan, and the pathogens were designated as Toyohashi and Ibaraki populations, respectively. They were maintained and multiplied in highly susceptible Chinese cabbage, *Brassica campestris* L. var. *chinensis* (L.) Makino, cvs. Granaat (ECD line '05') and Muso, or

turnip, *B. campestris* var. *rapifera* Metz, cv. Taibyohikari by using common method of Williams¹². The clubroot tissues were recovered from the inoculated Chinese cabbage or turnip plants, followed by crushing in sterilized distilled water in a petri dish with tweezers. Resting spores were easily suspended when clubbed tissues were severely rotted. Spore suspension was filtered through 8 layers of gauze, and adjusted to the concentration of 2×10^3 spores/ml, so as to contain one single spore in 0.5 μ l of the suspension. A 0.5 μ l drop of the suspension was placed on a piece of sterilized cover glass with a micropipette. The drop was checked at least three times under an inverted microscope to ensure that it carried exactly one spore. The drop was added with one microliter of sterilized distilled water in order to prevent the spore from desiccation. The cover glasses with drops were transferred to petri dishes with moistened filter paper until employed.

Inoculation to plant with single spore. Seeds of Chinese cabbage (cvs. Muso or Granaat) and turnip (cv. Taibyohikari) were surface-sterilized by immersing in 70% ethanol for ca. 1 min and subsequent 0.5% sodium hypochlorite for 30 min, and rinsed three times with sterilized distilled water. The seeds were placed in petri dishes with moistened filter paper and incubated at 25°C for 1 or 2 days. The cover glass carrying the single spore was placed on the surface of autoclaved soil with which a polycarbonate bottle (6×6×10 cm) was filled. The young seedling was placed over the drop on the cover glass, and covered with a small amount of potting soil,

* Faculty of Agriculture, Gifu University, Yanagido, Gifu 501-11, Japan 岐阜大学農学部

** Present address: National Institute of Agro-Environmental Science, Tsukuba 305, Japan 現在 : 農業環境技術研究所

named as the cover glass method. The bottles with the inoculated seedlings were covered with polypropylene lids for a week to keep humidity and to enhance infection with the pathogen. The seedlings were kept in a growth chamber at 25°C with a 16-hr photoperiod for 3 weeks. The plants were then transferred to another growth chamber maintained at 20°C at night and 25°C in the daytime, and grown for a further 5-weeks period. The gall formation on roots was graded with the scale from 0 (no clubs) to 3 (severe swelling on lateral and tap roots) according to Naiki *et al.*⁷⁾

Pathogenicity test. The host lines of European Clubroot Differential (ECD) set²⁾ were used to compare pathogenicity between the single spore isolates and their original populations. ECD lines 01 or 04 were not used because all these hosts had never been attacked by Toyohashi and Ibaraki populations in the preliminary experiments. Inoculum level obtained from the galls which had been formed with the single spore inoculation was not enough to use to test pathogenicity because a large amount of inoculum is required to inoculate 11 host lines of ECD. Therefore, multiplication of inoculum was conducted by using cover glass method in which inoculum was obtained by crushing the galls with a mortar and a pestle. Host plants were inoculated by modifying the technique known as 'slurry method'¹¹⁾. Inoculum for pathogenicity test was obtained by macerating the multiplied galls, filtering the suspension through 8 layers of gauze and centrifuging the filtrate¹²⁾. The spore suspension was mixed with autoclaved soil (1:2, w/w) to make slurry. The spore concentration in the slurry was adjusted to $0.3\text{--}3.0 \times 10^6/\text{ml}$. On each pot (5 cm in diam. and 10 cm high) containing autoclaved soil, a hole of 3 cm diam. and 2 cm deep was made with a centrifuge tube. Approximately 20 ml of the slurry was poured into

the hole in each pot, and 6 seedlings of one-day-old were immediately transplanted on the slurry. The pots were placed on trays filled with water up to 1–2 cm high and incubated on greenhouse benches. The number of plants per pot was thinned afterward to 5 plants. The test for each ECD line was replicated five times. The galls were graded 6 to 8 weeks after inoculation, as described in the preceding paragraph. The disease severity was calculated in the following. The number of plants in index 0 was multiplied by 0, in index 1 by 10, in index 2 by 80, and in index 3 by 100, and the sum was divided by the total number of plants.

RESULTS

Gall formation with single-resting-spore inoculation

Through thirteen trials of single spore inoculation, eight clubroot galls were successfully obtained (Table 1). Galls were formed in both Chinese cabbage and turnip, independently of the seedling age. Out of 8, half of the galls formed were rated as index 3, and the others showed less than 5 mm (Fig. 1). In the both cases more than one gall were developed on a plant.

Differences in pathogenicity of single-resting-spore isolates

Pathogenicity of five single-spore isolates multiplied was compared with the original populations (Table 2). In Toyohashi population, since the single-spore isolates were derived from the inoculum of either turnip or Chinese cabbage, the two hosts were employed for the corresponding comparison. All single-spore isolates (To-1–To-6) gave similar results to their original population for all ECD lines tested. Isolate Ib-1 from Ibaraki population, however, differed from its original popula-

Table 1. Clubroot formation in Chinese cabbage and turnip inoculated with single spore of Toyohashi population of *Plasmodiophora brassicae*

Host	Cultivar	Time of incubation ^{a)}	Replication	No. of plants					
				Tested	Dead ^{b)}	Disease index ^{c)}			
						0	1	2	3
Chinese cabbage	Granaat	24 hr	1	10	0	10	0	0	0
			1	9	0	7	2	0	0
			2	10	0	10	0	0	0
			3	13	0	12	0	0	1
			4	9	0	8	1	0	0
			5	9	1	8	0	0	0
			6	8	0	8	0	0	0
	Muso	48 hr	7	10	0	10	0	0	0
			1	8	0	7	0	0	1
			2	14	0	14	0	0	0
Turnip	Taibyo hikari	24 hr	3	12	0	10	1	0	1
			1	15	2	12	0	0	1
			2	10	0	10	0	0	0

a) Seeds were germinated in a moist petri dish at 25°C in the dark.

b) Non-emerging and dead plants.

c) Disease index was based on the scale of 0 (no clubs) to 3 (severe swelling on lateral and tap roots).

Table 2. Pathogenicity of single spore isolates and their original population of *Plasmodiophora brassicae* to European Clubroot Differential (ECD) hosts

ECD host	Toyohashi population						Ibaraki population	
	Turnip ^{a)}		Chinese cabbage ^{a)}				Chinese cabbage ^{a)}	
	To-1	Original	To-2	To-4	To-6	Original	Ib-1	Original
<i>Brassicae campestris</i>								
05	100	100	100	95	98	100	100	100
<i>B. napus</i>								
06	100	100	93	98	98	100	98	100
07	100	99	97	96	98	100	100	100
08	100	100	100	96	99	100	100	100
09	98	100	85	86	95	98	97	100
10	17	19	12	18	20	15	11	27
<i>B. oleracea</i>								
11	19	19	42	27	39	27	29	49
12	26	— ^{b)}	35	27	28	29	17	75
13	58	—	53	62	63	67	66	63
14	87	—	96	95	84	97	97	100
15	22	—	23	30	25	30	18	22

a) Inoculum tested was multiplied with turnip or Chinese cabbage.

b) Not tested.

Fig. 1. The galls formed on the roots of *Brassica campestris* var. *chinensis* cv. Muso by inoculation with single resting spore of *Plasmodiophora brassicae*. Right: disease index 3; left: index 1.

tion in pathogenicity; the isolate caused apparently lower disease to ECD line 12, although the other ECD lines the disease severity was similar.

DISCUSSION

Two technical problems may exist in single spore inoculation; one is to isolate single spores and the other to inoculate to host seedlings with the isolated spores. Reports^{6,8)} suggested the importance to use mature spores with high viability. Suzuki *et al.*⁹⁾ demonstrated that resting spores in rotten galls were highly germinative, and therefore such galls were employed in the

present study. In order to isolate single spores, Tinggal & Webster¹⁰⁾, Jones *et al.*⁶⁾ and Scott⁸⁾ used the special instruments, such as a dummy microscope objective lens on which a punch was mounted to cut and lift out a disc of agar with a single spore. In our study, only an inverted microscope and a micropipette are needed. The technique is simple and not laborious; at least 4 single spores per hour could be isolated. Our procedure is similar to the paraffin drop method proposed by Buczacki¹¹⁾, but paraffin has an inhibitory effect on germination and infection of spores, according to Tinggal and Webster¹⁰⁾.

The present cover glass method has an advantage that the seedlings employed rarely lose the single resting spores applied. The method gave the same levels of infection as Tinggal and Webster¹⁰⁾, and proved effective several times as much as Buczacki¹¹⁾, Jones *et al.*⁶⁾ and Scott⁸⁾. Furthermore, the method was applicable for efficient multiplication of the inoculum from small galls obtained by the single spore inoculation.

In Ibaraki population, the single spore isolate differed from its original population in pathogenicity to ECD line 12. Wit & Weg¹³⁾ and Jones *et al.*⁵⁾ suggested that a pathogen population from a field plot or even in a single clubroot gall was heterogeneous in pathogenicity. The studies of single spore isolation^{6,8,10)} clearly proved that the field population included some variations with different pathotypes. The present results confirmed the pathogenic heterogeneity in populations of *P. brassicae* in Japan.

Literature cited

1. Buczacki, S.T. (1977). Root infections from single resting spores of *Plasmodiophora brassicae*. Trans. Br.

- Mycol. Soc. 69: 328-329.
2. Buczacki, S.T., Toxopeus, H., Mattusch, P., Johnston, T.D., Dixon, G.R. and Hobolth, L.A. (1975). Study of physiologic specialization in *Plasmodiophora brassicae*: Proposals for attempted rationalization through an international approach. Trans. Br. Mycol. Soc. 65: 259-303.
 3. Dixon, D.R. (1986). Variation in *Plasmodiophora brassicae*. Ann. Appl. Biol. 94: 278-280.
 4. Ikegami, H. and Mukobata, H. (1990). Growth on medium of *Plasmodiophora brassicae* infected callus tissue (Studies on the clubroot of cruciferous plants IX) Res. Bull. Fac. Agr. Gifu Univ. 55: 1-14.
 5. Jones, D.R., Ingram, D.S. and Dixon, G.R. (1982). Factors affecting tests for differential pathogenicity in populations of *Plasmodiophora brassicae*. Plant Pathol. 31: 229-238.
 6. Jones, D.R., Ingram, D.S. and Dixon, G.R. (1982). Characterization of isolates derived from single resting spores of *Plasmodiophora brassicae* and studies of their interaction. Plant Pathol. 31: 239-246.
 7. Naiki, T., Kageyama, K. and Ikegami, H. (1978). The relation of spore density of *Plasmodiophora brassicae* Wor. to the root hair infection and club formation in Chinese cabbage (Studies on the clubroot of cruciferous plant II). Ann. Phytopathol. Soc. Jpn. 44: 432-439.
 8. Scott, E.S. (1985). Production and characterization of single-spore isolates of *Plasmodiophora brassicae*. Plant Pathol. 34: 287-292.
 9. Suzuki, K., Matsumiya, E., Ueno, Y. and Mizutani, J. (1992). Some properties of germination-stimulating factor from plants for resting spores of *Plasmodiophora brassicae*. Ann. Phytopathol. Soc. Jpn. 58: 699-705.
 10. Tinggal, S.H. and Webster, J. (1981). Technique for single spore infection by *Plasmodiophora brassicae*. Trans. Br. Mycol. Soc. 76: 187-190.
 11. Toxopeus, H. and Janssen, A.M.P. (1975). Clubroot resistance in turnip. II. The 'slurry' screening method and clubroot races in the Netherlands. Euphytica 24: 751-755.
 12. Williams, P.H. (1965). A system for the determination of races of *Plasmodiophora brassicae* that infect cabbage and rutabaga. Phytopathology 56: 624-626.
 13. Wit, F. and Weg, M., var der (1964). Clubroot-resistance in turnips (*Brassica campestris* L.). Euphytica 13: 9-18.

和 文 摘 要

景山幸二・上村順子・百町満朗：アブラナ科野菜根こぶ病菌の単一休眠胞子による簡便な接種法

アブラナ科野菜根こぶ病菌の単一の休眠胞子を受感性宿主に接種することにより発病させる簡便な技術を開発した。0.5 μ l 当たり1個、すなわち1 ml 当たり2000個の濃度になるように調整した休眠胞子懸濁液をマイクロピペットで0.5 μ l 取り、殺菌したカバーグラス上に滴下した。この液の中に1個の休眠胞子が含まれることを倒立顕微鏡で確かめ、カバーグラスを高圧蒸気滅菌した育苗培養土上に置き、2日間催芽させたハクサイの苗を液に接するように載せて軽く覆土し、人工気象器内で栽培した。この方法により高率に根こぶを形成させることができた。欧州判別品種を用いて単一休眠胞子由来株とその元株で病原性を比較したところ、両者が異なる場合があることが認められた。