European Journal of Plant Pathology **102:** 377–383, 1996.

Production, characterization and interaction of single-spore isolates of *Plasmodiophora brassicae*

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Accepted 27 November 1995

Key words: Brassica oleracea, B. napus, clubroot, single-spore isolate, induced resistance

Abstract

Out of 164 plants of clubroot-susceptible Chinese cabbage inoculated with single resting spores of *Plasmodiophora brassicae*, two plants developed clubroot symptoms. The two single-spore isolates (SSIs) extracted from these plants gave an identical reaction pattern on the European Clubroot Differential set (ECD) and seven doubled-haploid lines (DH-lines). Their reaction pattern differed from that of the original field isolate on four hosts: ECD hosts 06 and 07 were susceptible to the field isolate but resistant to both SSIs, while for DH-lines Bi and Pt the reverse was true. DH-line Pt was significantly less diseased by mixed inocula consisting of the field isolate and SSI-1 than by SSI-1 alone. It was concluded that the SSI-1 pathotype was a minor component of the field isolate, although it was isolated twice. The results also suggest that the alleviating effect of the field isolate in mixed inoculations with SSI-1 on DH-line Pt was due to induced resistance, rather than to competitive interactions.

Abbreviations: cv – cultivar; DH-line – doubled haploid line; ECD – European Clubroot Differential set; SSI – single-spore isolate.

Introduction

Natural populations of *Plasmodiophora brassicae* Woron., the causal agent of the clubroot disease of cruciferous crops, consist of mixtures of pathotypes [Haji Tinggal and Webster, 1981; Jones et al., 1982a]. Isolates consisting of only one pathotype are more suitable for genetic studies of the pathogen, and for the study of genes conferring resistance to host plants. Since the resting spores of *P. brassicae* are haploid [Tommerup and Ingram, 1971], the progeny of one single resting spore may be assumed to be genetically homogeneous.

As *P. brassicae* is an obligate parasite, isolates derived from a single resting spore (single-spore isolates, SSIs) can only be obtained by inoculating host plant tissue with isolated resting spores. Several methods have been used to perform single-spore inoculations [Buczacki, 1977; Haji Tinggal and Webster, 1981; Jones et al., 1982b; Scott, 1985; Schoeller and Grunewaldt, 1986; Schulte, 1994]. Varying proportions of single-spore inoculations by these authors resulted in diseased plants.

The single-spore inoculations mentioned above were made with resting spores from various natural populations. Inoculum consisting of resting spores extracted from the inoculated plants again induced clubroot symptoms on susceptible plants, showing that the life-cycle of the pathogen can be completed by genetically uniform isolates. Contrasting differential pathogenicity was found among the resulting SSIs, even among those derived from the same natural population [Haji Tinggal and Webster, 1981; Jones et al., 1982b; Scott, 1985; Schoeller and Grunewaldt, 1986; Schulte, 1994]. This indicated that various pathotypes of *P. brassicae* are capable of survival as uniform isolates.

With the aim of obtaining genetically uniform isolates of *P. brassicae* for the study of resistance in *Brassica oleracea*, inoculations with single resting spores of a Dutch population of the pathogen were

performed. Here the production and characterization of SSIs are reported. The SSIs displayed a differential pathogenicity not expressed by the original field isolate. Since suppression of differential pathogenicity in heterogeneous inocula can have serious consequences especially for resistance breeding, this phenomenon was further studied in mixed inoculations of one SSI and the field isolate on several host accessions.

Materials and methods

Pathogen

A field isolate of *P. brassicae* was obtained from clubs of an unknown clubroot-susceptible cauliflower cultivar grown on an infested field of the Experimental Station Brabant at Breda, The Netherlands, and maintained on chinese cabbage (*B. rapa*) cv Granaat. This host cv has been widely used in clubroot research and is not known to carry resistance to this disease. The field isolate was characterized as ECD 16/3/30 [Buczacki et al., 1975; Voorrips and Visser, 1993].

Plant material

The 15 components of the European Clubroot Differential set [ECD; Buczacki et al., 1975] were obtained from H. Toxopeus (CPRO-DLO). ECD-host 08 segregated for resistance to the field isolate; for the tests reported here a uniformly resistant inbred line of this host was used.

Seven doubled-haploid lines (DH-lines) were obtained through microspore culture [Duijs et al., 1989]. DH-line Gr was derived from broccoli cv Greenia (Hammenhögs Frö AB, Hammenhög, Sweden), DH-line O7 from broccoli line OSU CR-7 [Baggett and Kean, 1985] and DH-line Pt from curly kale cv Petibor-F1 (Bejo Seeds b.v., Warmenhuizen, the Netherlands). The other DH-lines were derived from cabbage accessions: lines Bi and Bö from lines selected by I.R. Crute (HRI, Wellesbourne, U.K.) from the landraces Bindsachsener and Böhmerwaldkohl; line Ch from a cross involving line 8–41 [Chiang and Crête, 1970]; line La from line Larson 8353 T [Nieuwhof and Wiering, 1963]. Cabbage cv Septa (Bejo Seeds b.v.), equivalent to ECD host 14, was used as a susceptible control.

Single-spore inoculations

Suspensions of resting spores of the field isolate of *P. brassicae* were prepared according to Voorrips and Visser [1993]. Each suspension was used during a period of 2–3 weeks and kept at 4 °C. Prior to use, an aliquot of suspension was diluted to a density of about 500 spores·ml^{-1}. A 2 μl droplet of the diluted suspension was pipetted on a microscope slide disinfected with 80% ethanol. The droplet was scanned in overlapping parallel lanes at 300 × magnification. If one or zero resting spores were observed, the droplet was scanned again. Putative resting spores were re-examined at 450 × magnification. Droplets containing one or zero resting spores were used for single-spore or mock inoculations respectively. A 5 to 8 days old seedling of chinese cabbage (*B. rapa*) cv Granaat (ECD-host 05) was placed with its roots on the microscope slide to adsorp the droplet. Subsequently the seedling was transferred to a 1.5 ml eppendorf centrifuge tube, and 1 ml of sterile water was poured over the slide into the tube. After the seedlings had been incubated 48 h in a climate room (23 °C, 80 μE·m^{-2}·s^{-1}, 16 h day), they were potted in sterile compost in a glasshouse (18 °C). As a check for contamination, uninoculated seedlings were planted between the seedlings inoculated with one resting spore. The seedlings with mock inoculations were planted separately. After seven weeks the symptoms were evaluated, and clubs were harvested for propagation.

Resistance tests and propagation of isolates

For resistance tests and propagation of isolates the pipette inoculation method was used [Voorrips and Visser, 1993]. Symptoms were evaluated after six weeks on a scale of 0–3 according to Buczacki et al. [1975]. A disease index was calculated as the average symptom grade divided by 3, to yield a value between 0 (no symptoms) and 1 (all plants with severe symptoms).

Six tests were performed, designated A to F. The treatments (combinations of host accession and inoculum) used in each test are mentioned in Tables 1 to 5. Each treatment was tested in at least two replicates of six pots, the exact number of replicates depending on the amount of seed available. The non-parametric Kruskal-Wallis statistic [Siegel, 1956] calculated from the symptom grades of the individual plants was used to test for differences between treatments within each accession. Where this statistic proved significant ($P < 0.05$), pairwise comparisons between treatments within each accession were made using the non-parametric Mann-Whitney (or Wilcoxon) U-test [Siegel, 1956].

Propagation of isolates was carried out on chinese cabbage (*B. rapa*) cv Granaat. Clubs were harvested, washed under tap water and surface-sterilized (15 s in 70% ethanol, 20 min in 0.5% NaOCl, six rinses in sterile water) before storage or further use.

Results

Production of single-spore isolates

Over a period of two months, 164 single-spore inoculations were performed. Only two plants inoculated with a single spore developed symptoms, both in the most severe grade. None of the 161 mock inoculated plants (inoculated with droplets in which no resting spore was observed) or of the 160 control plants separating the inoculated plants became diseased. The SSIs in the two diseased plants were designated SSI-1 and SSI-2. SSI-1 was propagated once and SSI-2 twice to produce sufficient inoculum for further experiments.

Characterization of field and single-spore isolates

With the 14 ECD hosts and seven DH-lines tested in experiments A and B, no differences in reaction were observed between SSI-1 and SSI-2 (Tables 1 and 2). Two ECD hosts (ECD06 and ECD07) were susceptible to the field isolate and resistant to the SSIs, while for two DH-lines (Bi and Pt) the reverse was the case, indicating contrasting differential pathogenicity between the field isolate and the SSIs. In the period between experiments A and B the field isolate and both SSIs were propagated on *B. rapa* cv Granaat. Host ECD04 germinated erratically in test B and the test results obtained with this host were not considered to be reliable.

Experiments with mixtures of field isolate and SSI-1

In experiment D, mixtures of the field isolate and SSI-1 in ratios of 1:4, 1:1 and 4:1 with a fixed total inoculum density of $2 \cdot 10^7$ spores·plant^{-1} were compared with the pure inocula at the same and at half the density (Table 3). In experiment C, only the 1:1 mixture was compared with both pure isolates, yielding very similar results (not shown). ECD06 and ECD07 were fully susceptible to all inocula containing the field isolate, and highly resistant to pure SSI-1 at both inoculum densities. DH-lines Bi and Pt showed partial susceptibility to all inocula containing SSI-1. The inoculum mixture containing 20% SSI-1 caused less severe symptoms on these lines than inocula with higher proportions of SSI-1.

In experiments E and F, mixed inocula of the field isolate and SSI-1 were compared with inocula consisting of the same absolute amount of either field isolate or SSI-1 (Tables 4 and 5). Table 4 shows the results with cv Septa and DH-line Pt; DH-line Bi was not included due to an insufficient supply of seed. The presence of the field isolate reduced the severity of disease in DH-line Pt, but not in cv Septa, compared to the corresponding inoculum without field isolate at all levels of SSI-1 tested. The disease severity of DH-line Pt inoculated with pure SSI-1 was not significantly related to inoculum density, although the least symptoms were observed at the lowest inoculum density. From Table 5, it is apparent that ECD06 and ECD07 were severely affected by all inocula containing even a hundredfold reduced amount of the field isolate. ECD06 showed slightly reduced symptoms when 99% of the inoculum consisted of SSI-1; this reduction was not statistically significant ($P > 0.05$).

Discussion

Production of single-spore isolates

Two out of 164 single-spore inoculations produced clubroot symptoms on the susceptible host cv Granaat. This demonstrated that no cooperative action of different pathotypes is required for successful infection. The success rate was comparable to most of the results reported before [Buczacki, 1977; Haji Tinggal and Webster, 1981; Jones et al., 1982b; Scott, 1985], but was significantly smaller than the 8% and 66% reported by Schoeller and Grunewaldt [1986] and Schulte [1994] respectively. Differences in success rate may be caused by the condition of the inoculum, as well as by different inoculation procedures. Attempts to quantify the proportion of infecting resting spores from the same batch of field isolate as used in this study were made earlier [Voorrips, 1995]. The estimated proportions varied from $3 \cdot 10^{-5}$ to $6 \cdot 10^{-4}$ in different experiments using the pipette inoculation method. The rate of infection by individual spores was therefore much higher with the single-spore than with the pipette inoculation method, showing that different inoculation procedures may indeed cause large differences in success rate.

Table 1. Reactions of the European Clubroot Differential set (ECD; Buczacki et al., 1975) inoculated with two single-spore isolates (SSI-1 and SSI-2) of *Plasmodiophora brassicae* and the original field isolate (F.I.)

Host accession	F.I.[1]		F.I.[2]	SSI-1[2]	SSI-2[2]			
	pl[3]	D.I.[3]	pl	D.I.	pl	D.I.	pl	D.I.
ECD01	35	0.00	36	0.00	34	0.00	35	0.00
ECD02	34	0.00	27	0.00	32	0.00	31	0.00
ECD03	33	0.00	27	0.00	34	0.00	32	0.00
ECD04	38	0.00	–[4]	–	–	–	–	–
ECD05	37	1.00	39	0.98	36	0.98	41	1.00
ECD06	40	1.00	33	1.00	35	0.00	36	0.00
ECD07	33	0.98	36	1.00	36	0.01	36	0.00
ECD08[5]	39	0.00	36	0.00	34	0.00	36	0.00
ECD09	36	0.00	36	0.01	36	0.00	35	0.00
ECD10	35	0.00	35	0.04	36	0.04	36	0.09
ECD11	24	0.13	32	0.53	33	0.28	34	0.35
ECD12	36	0.96	24	0.99	31	0.98	25	0.99
ECD13	36	1.00	35	1.00	35	1.00	35	1.00
ECD14	21	1.00	29	1.00	43	1.00	40	1.00
ECD15	36	0.52	28	0.18	31	0.48	27	0.59

[1] Earlier test results with the field isolate [Voorrips and Visser, 1993], included here for comparison.
[2] Results of experiment B.
[3] pl: number of plants assessed; D.I.: disease index (see Materials and methods).
[4] –: no results due to germination problems.
[5] The original ECD08 segregated for resistance to the field isolate. The results in this table were obtained with an inbred line selected from the original accession.

Table 2. Reactions of seven doubled-haploid lines of *Brassica oleracea* inoculated with two single-spore isolates (SSI-1 and SSI-2) of *Plasmodiophora brassicae* and the original field isolate

Host accession	Field isolate				SSI-1				SSI-2			
	Exp. A		Exp. B		Exp. A		Exp. B		Exp. A		Exp. B	
	pl[1]	D.I.[1]	pl	D.I.	pl	D.I.	pl	D.I.	pl	D.I.	pl	D.I.
DH-line Bi	19	0.09	18	0.00	33	0.41	21	0.73	29	0.68	17	0.84
DH-line Bö	12	0.14	11	0.00		nt[2]	11	0.06		nt	12	0.00
DH-line Ch	27	0.00	14	0.00	13	0.00	16	0.00	15	0.00	16	0.02
DH-line Gr	23	1.00	18	1.00		nt	15	1.00		nt	18	1.00
DH-line La	38	0.04	18	0.00		nt	18	0.11		nt	17	0.12
DH-line O7	15	0.02	11	0.00	18	0.00	16	0.00	17	0.00	17	0.00
DH-line Pt	23	0.03	11	0.03	27	0.46	17	0.57	28	0.58	18	0.61

[1] pl: number of plants assessed; D.I.: disease index (see Materials and methods).
[2] nt: not tested.

Comparison of host specificity of field and single-spore isolates

No differences were observed between the two SSIs produced in this study in differential pathogenicity to all 21 hosts tested. Two major differences between both SSIs and the field isolate were found: ECD hosts 06 and 07 were susceptible to the field isolate but resistant to the SSIs, while remarkably for DH-lines Bi and Pt the reverse was true. The first type of difference was described before for other SSIs of *P. brassicae* [Haji Tinggal and Webster, 1981; Jones et al., 1982b; Scott, 1985; Schoeller and Grunewaldt, 1986; Schulte, 1994]. However, the second type of difference observed here, where a SSI is pathogenic on a host resistant to the original field isolate, was only described by Jones et al. [1982b] and Schoeller and Grunewaldt [1986], although the latter authors did not confirm their result in a separate experiment.

Table 3. Disease indices of *Brassica* accessions inoculated with mixtures of single-spore isolate SSI-1 and the original field isolate of *Plasmodiophora brassicae*

Host accession	Inoculum (10^7 spores·plant^{-1}) Field	1.0	2.0	1.6	1.0	0.4	0.0	0.0
	SSI-1	0.0	0.0	0.4	1.0	1.6	2.0	1.0
Septa		1.00[1]	1.00	1.00	1.00	1.00	1.00	1.00
		(31)	(28)	(36)	(36)	(36)	(27)	(29)
ECD06		1.00 b	1.00 b	1.00 b	1.00 b	1.00 b	0.01 a	0.04 a
		(29)	(30)	(35)	(35)	(31)	(30)	(28)
ECD07		1.00 b	1.00 b	1.00 b	1.00 b	1.00 b	0.00 a	0.00 a
		(24)	(22)	(30)	(31)	(30)	(24)	(24)
DH-line Bi		0.00 a	0.01 a	0.16 b	0.31 bc	0.43 cd	0.49 d	0.39 cd
		(24)	(24)	(27)	(29)	(25)	(19)	(23)
DH-line Pt		0.04 a	0.03 a	0.65 b	0.70 bc	0.84 c	0.77 bc	0.85 c
		(19)	(20)	(31)	(30)	(29)	(19)	(20)

[1] The number of plants tested is indicated within brackets. The occurrence of letters behind the disease indices in a line indicates the presence of significant ($P < 0.05$) inoculum effects. Within the same line disease indices followed by the same letter indicate that the effects of the corresponding inocula were not significantly different ($P \geq 0.05$).

Table 4. Disease indices of two *Brassica oleracea* accessions inoculated with mixtures of single-spore isolate SSI-1 and the original field isolate of *Plasmodiophora brassicae*

Host accession	Inoculum (10^7 spores·plant^{-1}) Field	2.0	0.0	1.6	0.0	1.8	0.0	1.98	0.00
	SSI-1	0.0	2.0	0.4	0.4	0.2	0.2	0.02	0.02
	Exp.								
Septa	E	1.00[1]	1.00	1.00	0.96	1.00	0.95	1.00	0.94
		(36)	(42)	(37)	(40)	(47)	(39)	(44)	(40)
	F	1.00	1.00	0.99	0.99	1.00	0.99	1.00	1.00
		(22)	(24)	(24)	(24)	(27)	(28)	(27)	(29)
DH-line Pt	E	0.04 a	0.75 c	0.48 bc	1.00 d	0.21 ab	0.67 c	0.02 a	0.21 ab
		(15)	(12)	(20)	(9)	(8)	(14)	(16)	(14)
	F	0.00 a	0.78 d	0.33 c	0.61 d	0.29 bc	0.70 d	0.12 ab	0.59 cd
		(9)	(9)	(11)	(11)	(7)	(10)	(14)	(9)

[1] As in Table 3.

Interaction of field and single-spore isolates

Tests E and F with inocula consisting of mixtures of SSI-1 and field isolate showed that DH-line Pt still reacted differently with mixtures consisting of only 10% SSI-1 compared with the pure field isolate (Table 4). This suggests that the SSI-1 pathotype is not a major component of the field isolate. The fact that this pathotype was obtained as a SSI in both cases is therefore hard to explain. Either it can be considered as an improbable coincidence, or as evidence for some kind of selection for specific pathotypes operating during the SSI extraction process. One can speculate for example that not all pathotypes of *P. brassicae* in the field isolate are able to complete their life-cycle in a homokaryotic and homozygous condition. More SSIs from the same field isolate are needed to substantiate this finding.

DH-line Pt was partially susceptible to SSI-1 at inoculum densities down to $2 \cdot 10^5$ spores·plant^{-1} or less (Table 4). Addition of the field isolate to a final density of $2 \cdot 10^7$ spores·plant^{-1} resulted in clearly reduced symptom development. This shows that some other

Table 5. Disease indices of three *Brassica* accessions inoculated with mixtures of single-spore isolate SSI-1 and the original field isolate of *Plasmodiophora brassicae*

	Inoculum (10^7 spores·plant^{-1})								
	Field	0.0	2.0	0.4	0.4	0.2	0.2	0.02	0.02
Host accession	SSI-1	2.0	0.0	1.6	0.0	1.8	0.0	1.98	0.00
	Exp.								
Septa	E	1.00[1]	1.00	0.99	0.97	1.00	0.96	1.00	0.97
		(42)	(36)	(37)	(35)	(32)	(33)	(42)	(39)
	F	1.00	1.00	0.95	0.98	1.00	0.98	1.00	0.97
		(24)	(22)	(14)	(19)	(15)	(20)	(18)	(21)
ECD06	E	–[2]	1.00	1.00	1.00	1.00	1.00	0.88	0.98
			(11)	(16)	(14)	(19)	(17)	(17)	(18)
	F	0.00 a	1.00 b	1.00 b	1.00 b	1.00 b	1.00 b	0.90 b	1.00 b
		(16)	(11)	(16)	(16)	(15)	(16)	(14)	(18)
ECD07	E	–[2]	1.00	1.00	1.00	1.00	1.00	0.98	1.00
			(17)	(19)	(18)	(18)	(18)	(18)	(18)
	F	0.02 a	1.00 b	1.00 b	1.00 b	1.00 b	1.00 b	1.00 b	1.00 b
		(16)	(15)	(14)	(17)	(18)	(18)	(17)	(16)

[1] As in Table 3.
[2] no results due to experimental mistake.

pathotype present in the field isolate, non-pathogenic on this line, either competed very successfully with SSI-1 for infection sites or other limited host resources, or rapidly induced resistance in this host genotype. A similar conclusion was reached by Jones et al. [1982b].

On ECD06 and ECD07 no alleviating effect of SSI-1 was observed (Table 5). Moreover, Voorrips [1995] showed that interactions between spores, competitive or otherwise, on a susceptible host do not have a large influence on the rate of infection by individual spores. Therefore the competition by non-pathogenic pathotypes for limited host resources is not likely to be the major cause of the interaction between the field isolate and SSI-1 when inoculated on DH-line Pt. This then leaves the possibility of induced resistance as an explanation of the observed effect. Reports of resistance to pathogens induced in plants by non-pathogenic micro-organisms are numerous [Madamanchi and Kuć, 1991]. In contrast to reported forms of induced resistance, where a lag period is required between application of the inducing and the pathogenic organism, the resistance described in this paper is induced by simultaneous inoculation with the inducing and the pathogenic pathotype. This may be explained by the fact that early contact between *P. brassicae* and its *Brassica* hosts in the root hair stages of infection is non-specific, whereas the specific resistant reaction develops later, in the root cortex [Voorrips, 1992].

Consequences of putative induced resistance

The possible occurrence of resistance induced by non-pathogenic pathotypes has important consequences for the strategy of breeding for clubroot resistance. The common practice of mixing isolates to screen breeding material for a broad resistance to clubroot may in fact reduce the level of infection compared to separate inoculation of the isolates, rather than enhance it. It will therefore be necessary to test also with separate isolates. Further, if a host genotype is resistant to a field isolate, this may be caused by induced resistance. Such resistance may break down by disappearance of inducing pathotypes as well as by the emergence of new pathotypes. Therefore, induced resistance may be less durable than constitutively expressed forms of resistance. On the other hand, cultivars capable of expressing induced resistance could conceivably benefit from the artificial application of an inducing pathotype to the field. A first priority for further studies on this subject is the isolation of resistance inducing SSIs.

Acknowledgements

The technical assistance of M. Zevenbergen, G.P. Terwoert and H.J. Kanne is gratefully acknowledged. Also the demonstration by U. Schulte of his single-spore inoculation technique was very helpful. Thanks are

due to Dr W.H. Lindhout, Prof Dr Ir P.J.G.M. de Wit and Prof Dr Ir J.E. Parlevliet of Wageningen Agricultural University for their comments and suggestions.

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