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Detection of *Plasmodiophora brassicae* by PCR in naturally infested soils

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Abstract

A nested polymerase chain reaction (PCR) method was developed for detection of DNA from *Plasmodiophora brassicae* in naturally infested field soil samples. The target sequences 389 bp and 507 bp were amplified from Swedish populations of *P. brassicae.* The protocols described enabled detection of DNA in various soil classes with an inoculum level of *P. brassicae* corresponding to a disease severity index (DSI) higher than 21 in a greenhouse bioassay. Three sequenced Swedish *P. brassicae* isolates had identical sequence in the 18S/ITS 1 region, but differed by a few nucleotides from an isolate sequenced in the UK. The results indicate that the primers used are general for *P. brassicae*, and consequently the nested PCR assay has a potential to be developed as a routine diagnostic test.

Introduction

Plasmodiophora brassicae, the cause of the clubroot disease of crucifers, belongs to the Plasmodiophorids, a group of obligate, intracellular plant parasites. Recent phylogenetic analyses suggest closest taxonomic affinity with some protozoa (Protoctista) (Braselton, 1995; Castlebury and Domier, 1998; Ward and Adams, 1998). Clubroot was considered the economically most important disease of cultivated crucifers by Karling (1968) and it is widely disseminated throughout the growing districts of oilseed rape and vegetable brassicas (CMI Maps, 1977; Robak, 1991; Linnasalmi and Toiviainen, 1991; Engqvist, 1994; EPPO, 1999). In a Swedish survey, clubroot was detected in 78% of 190 fields assayed in a spring-rape growing area (Wallenhammar, 1996). The longevity of the resting spores that are liberated in large numbers into the soil when infected roots decompose, makes control of the disease difficult (Wallenhammar, 1996). Control measures in cabbage crops have included application of different chemical substances, but effective and environmentally acceptable means for direct control are still lacking (Dixon et al., 1998). In oilseed crops, neither resistant cultivars nor effective means of chemical control are available. Much attention has been directed towards avoiding cultivation of oilseed rape in contaminated soil. Clubroot infested fields may be identified by soil bioassays (Brokenshire and Lewis, 1981; Wallenhammar, 1996), but these take 5–6 weeks to carry out. There is consequently great interest in developing rapid, sensitive, and reliable methods for detecting the presence of *P. brassicae* in soil, and this is further exaggerated by the introduction of partly resistant cultivars of spring oilseed turnip rape (*Brassica campestris).* Since these cultivars are preferably grown on fields where soil infestation level gives a disease severity index (DSI) of less than 10 (Wallenhammar et al., 2000), there is an urgent necessity for an effective routine detection method.

Various methods based on immunological procedures have been tested. Lange et al. (1989) detected *P. brassicae* in infected roots using dot immunobinding, and White and Wakeham (1995) detected resting

spores in artificially and naturally infested soils by indirect immunoflorescence and by indirect enzyme-linked immunosorbent assays. However, none of these methods have been fully developed for use in routine testing of field soils. Another approach has used techniques based on the polymerase chain reaction (PCR). Ito et al. (1997) identified a single copy DNA sequence unique to *P. brassicae* and designed specific primers for amplifying the target sequence that is unique to this pathogen. Recently, Faggian et al. (1999) designed primers from the ribosomal repeat and internal transcribed spacer (ITS) region, the partial sequence of 18S, ITS-1, 5.8S, ITS-2 and partially 28S regions.

Faggian et al. (1999) were able to detect *P. brassicae* in artificially infested soil samples, indicating that it is possible to overcome problems related to applying PCR to soil or sediment samples, through extensive purification of DNA, and optimizing the PCR cycling parameters (Elväng, 1998). Suitable technology has also been worked out for monitoring specific bacteria (Steffan et al., 1988; Bej et al., 1990; Möller and Harling, 1995). There are, furthermore, reports on detection of *Helmintosporium solani, Colletotrichum coccoides* and *Streptomyces scabies* in artificially infested soil samples (Cullen et al., 1999) and on detection of *Spongospora subterranea* in soil samples (Bulman and Marshall, 1999).

The aim of the present study was to develop a rapid and reliable detection method for evaluating soil inoculum levels of *P. brassicae* in naturally infested soils. We also report on the identification of Swedish *P. brassicae* isolates by sequencing the 18S/ITS 1 region.

Materials and methods

DNA extraction from soil

Soil samples were collected from 15 fields where severe attacks of clubroot were detected in spring oilseed brassicas and brussel sprouts in either 1997 or 1998. Samples from infected fields and from uninfected fields (Table 1) were collected in the area of Örebro in the central part of Sweden (59° N) during November 1998 and stored in polyethylene bags at 4 °C. About 3 liters of soil was collected. Soil samples were air dried at room temperature (20 °C), and pulverized by hand or a pestle, thoroughly mixed in a bucket, before a sub-sample of 500 g was withdrawn. Infected roots to which soil was attached were also collected and stored frozen. Direct DNA extraction was done according to the protocol of Volossiouk et al. (1995) where a soil sample of 250 mg was ground with liquid nitrogen combined with sodium dodecyl sulfate (SDS), and extracted with phenol. DNA losses due to adsorption or degradation are often minimized through addition of a nucleic acid carrier or other polyvalent polymers. In this case, skim milk powder at a concentration of either $4\,g\,l^{-1}$ or of $40\,g\,l^{-1}$ water was used. These concentrations are referred to as LCSM (low concentration of skim milk) and HCSM (high concentration of skim milk).

Table 1. Infestation according to bioassay of sampled soils, values of selected physiochemical soil properties, and information on the *Brassica* crops cultivated on the fields sampled. Duncan's multiple range test ($P > 0.05$) is used to compare soil infestation levels. Those followed by the same small letter are not significantly different

No.	Experiment site	Infected plants (DSI)	Infected plants (%)	Ca[1]	Mg[1]	pH (water)	Sand[2]	Humus content[2]	CEC[3]	Clay content[2]	Previous *Brassica* crop
2	Tybble	63cd	75bc	170	14	6.5	32	2.7	14.8	17	*Brassica napus*
3	Åkerby	88a	100a	350	38	6.4	39	11.9	25.6	9	*Brassica oleracea*
5	Åkerby	0g	0f	160	17	6.2	15	3.2	14.2	14	Control (clay)
6	Segersjö	49de	68cd	210	20	6.9	36	3.4	15.4	17	*Brassica campestris*
7	Ytterby	84a	96a	95	4.2	6.3	62	2.1	9.8	8	*B. napus*
10	Almby	21f	35e	170	37	6.6	23	0.4	16.6	29	*B. napus*
11	Glans-Hammar	68bc	88ab	160	22	5.9	41	3.0	17.1	22	*B. napus*
15	Åkerby	0g	0f	94	6.8	5.8	37	2.7	11.1	9	Control (fine sand)
16	Röcklinge	88a	98a	150	20	7.1	8	2.8	15.4	27	*B. campestris*
17	Ekeby	8gf	13f	240	9.9	6.2	16	3.0	15.2	18	*B. oleracea*

[1]mg/100 g soil.
[2]percent.
[3]Meq/100 g.

DNA extraction from infected roots

Extraction of DNA was carried out by the boiling method (Ward and Adams, 1998). Infected root that had been stored frozen, were cut into 2 cm pieces and placed in a screw top Eppendorf tube. 200 µl of 1 M Tris–HCl was added and the mixture crushed using a metal rod. The tube was placed in boiling water for 10 min, then quickly cooled in ice. The resulting liquid was twice centrifuged at 2500 rpm for 4 min and then stored at −20 °C. The amount of DNA was measured spectrophotometrically at 260 nm, using the relationship 50 µg ml^{-1} DNA equals A_{260} of 1.0. As the first step in designing a detection method, DNA was amplified from infected roots using the primers PBTZS-3 and PBTZS-4. Amplification products were generated with a DNA amount of 260 ng (Table 4). DNA extract from roots grown in soil number 2 and 7 were used as control templates.

PCR amplification of soil DNA extracts

A nested PCR method for detection of *P. brassicae* DNA was based on the primers (PBTZS-3 and PBTZS-4) designed by Ito et al. (1997) for the isopentyltransferase gene. Two sets of nested primers (PBAW-10 and PBAW-11) and (PBAW-12 and PBAW-13) were designed with Primer Select from DNA-Star package (DNA Star Inc., Madison.WI, USA) using Ito's sequence D85819 (Ito et al., 1997). Our method was later compared with another recently described method (Faggian et al., 1999). Their primers PbITS1, PbITS2, PbITS6 and PbITS7 were designed from the ITS1, 5.8S and part of the 18S and ITS2 regions. All primers were supplied by Life Technologies, Täby, Sweden and are described in Table 2.

PCR conditions were (i) 3 min at 94 °C, 40 cycles of 1 min at 94 °, 2 min at 50 °, 1 min at 72 ° and 5 min at 72 ° for Ito's method. (ii) 10 min at 94 °C, 45 cycles of 1 min at 94 °, 1 min at 60 °, 2 min at 72 ° and 5 min at 72 ° for Faggian's method. All amplifications were performed on a Perkin Elmer Cetus DNA Thermal Cycler with the following reaction mix: 1X PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM each of dNTP and 1% formamide, 1 µM of each primer, 20 µl soil DNA extract and 2.5 U AmpliTaq Gold DNA polymerase (Perkin Elmer). Normal AmpliTaq DNA polymerase (Perkin Elmer) was used in one experiment.

In the nested PCRs, 1 µl of the first amplification was used as a template for the second PCR. Aerosol resistant tips were used and the PCR mixture was prepared in a laminar flow cabinet separated from the PCR machine and gel electrophoresis. Ten µl of the PCR reaction was analysed on a 1.5% agarose gel stained with about 30 ng ml^{-1} EtBr, visualized under 302 nm UV light and photographed with a Polaroid system.

Sequencing

With Qiaquick PCR purification kit (Qiagene Ltd. Crawley, West Sussex, UK), 40 µl of a PCR reaction from soils 2, 11 and 16 respectively, was purified using 200 µl buffer PB and eluted with 50 µl water. The sequencing reaction was performed with BigDye terminator Cycle Sequencing Ready reaction kit (Applied Biosystems, Stockholm, Sweden) using 2 µl purified PCR product, 1.6 pmol sequencing primer and 4 µl

Table 2. Characteristics of primers used in the various PCR reactions

Primer	Sequence 5′→3′	Length of amplified *P. brassicae* DNA	Reference
PBTZS-3	CCACGTCGATCACGTTGCAAT		Ito et al. (1997)
PBTZS-4	CCTGGCGTTGATGTACTGGAA	398bp*	Ito et al. (1997)
PBAW-10	CCCCGGGGATCACGATAAATAACA		This report
PBAW-11	GGAAGGCCGCCCAGGACTACC	774bp**	This report
PBAW-12	GCCGGCCAGCATCTCCAT		This report
PBAW-13	CCCCAGGGTTCACAGCGTTCAA	1085bp**	This report
PbITS-1	ACTTGCATCGATTACGTCCC		Faggian et al. (1999)
PbITS-2	GGCATTCTCGAGGGTATCAA	1086bp**	Faggian et al. (1999)
PbITS-6	CAACGAGTCAGCTTGAATGC		Faggian et al. (1999)
PbITS-7	TGTTTCGGCTAGGATGGTTC	507bp**	Faggian et al. (1999)

*Predicted from sequence of *P. brassicae*, accession no D85819 (Ito et al., 1997).
**Predicted from sequence of *P. brassicae*, accession no Y12831 (Ward and Adams, 1998).

reaction mix, in a 10 µl total volume. The sequences were read with an ABI Prism 310 sequenator. The positions of 18S and ITS1 were predicted from sequence comparisons with other species in the database (NCBI) especially the closely related *S. subterranea* (accession no. AF102819).

Assessing the infection capacity of naturally infested soil samples by bioassay

The level of *P. brassicae* soil infestation in the soils sampled was measured by assessing the number of bait plants infected according to the greenhouse bioassay described by Wallenhammar (1996), with the exception that a smaller pot size, Vefi (Larvik, Norway), 5 × 5 × 5 cm, was used. Infected plants were scored according to a disease index (Wallenhammar et al., 2000). Measurements of selected physiochemical parameters in the soils were performed by AgroLab Scandinavia AB, Kristianstad, Sweden.

Results

Assessing the infection capacity by bioassay

The levels of *P. brassicae* soil infestation are presented in Table 1. Disease severity indices (DSI) ranged from 0 to 88. For the subsequent PCR amplification, six soils were chosen to represent the various infection levels: soils 3, 6, 10, 11, 16 and 17 along with the two negative control soils, 5 and 15.

DNA content in soil and root extracts

The amount of DNA in soil extracts ranged between 3.9 and 17.4 µg ml^{-1} when extracted with LCSM and between 0.9 and 2.9 µg ml^{-1} when extracted with HCSM. The amount measured in a root extract from soil 7 was 1323.8 µg ml^{-1} (Table 3). A higher concentration was regularly extracted with the low level of skim milk powder.

Sensitivity of the methods

The sensitivity of the primers was measured by making PCRs on 10-fold serial dilutions of the root extract,

Table 3. DNA concentrations in soil and root extracts measured spectrophotometrically at 260 nm

Extraction material	Extracted with 4 g l^{-1} skim milk (µg/ml)	Extracted with 40 g l^{-1} skim milk (µg/ml)
Soil No. 3	17.4	
Soil No. 5	6.9	
Soil No. 15	3.9	
Soil No. 17	5.4	
Soil No. 6	5.4	3.2
Soil No. 10	3.9	0.9
Soil No. 11	6.7	2.9
Soil No. 16	7.7	1.7
Soil from infected roots, soil No. 2		10.8
Root extract, grown in soil No. 7	1323.8*	

*Extracted with the boiling method.

Table 4. Sensitivity of different combinations of primers. PCR amplifications of diluted extracts from galls on roots infected with *P. brassicae.* The undiluted root extract has a total amount of DNA of 26 µg ml^{-1}

Dilution	DNA content	Primer pairs used			
		PBTZS-3,4[1]	PBAW-12,-13; PBTZS-3,-4[2]	PBAW-10,-11; PBTZS-3,-4[3]	PbITS-1,-2; PbITS-6,-7[4]
10^0	26 µg	Yes	Yes	Yes	Yes
10^{-1}	2.6 µg	Yes	Yes	Yes	Yes
10^{-2}	260 ng	Yes	Yes	Yes	Yes
10^{-3}	26 ng	No	Yes	Yes	Yes
10^{-4}	2.6 ng	—	No	Yes	Yes
10^{-5}	260 pg	—	No	Yes	Yes
10^{-6}	26 pg	—	No	No	No
10^{-7}	2.6 pg	—	No	No	No

[1]Main primers PBTZS-3,-4 (Ito et al., 1997).
[2]Main primers PBAW-12,13 (this report), nested primers PBTZS-3,-4.
[3]Main primers PBAW-10,-11 (this report), nested primers PBTZS-3,-4.
[4]Main primers PbITS-1,-2 (Faggian et al., 1999), nested primers PbITS-6,-7 (Faggian et al., 1999).

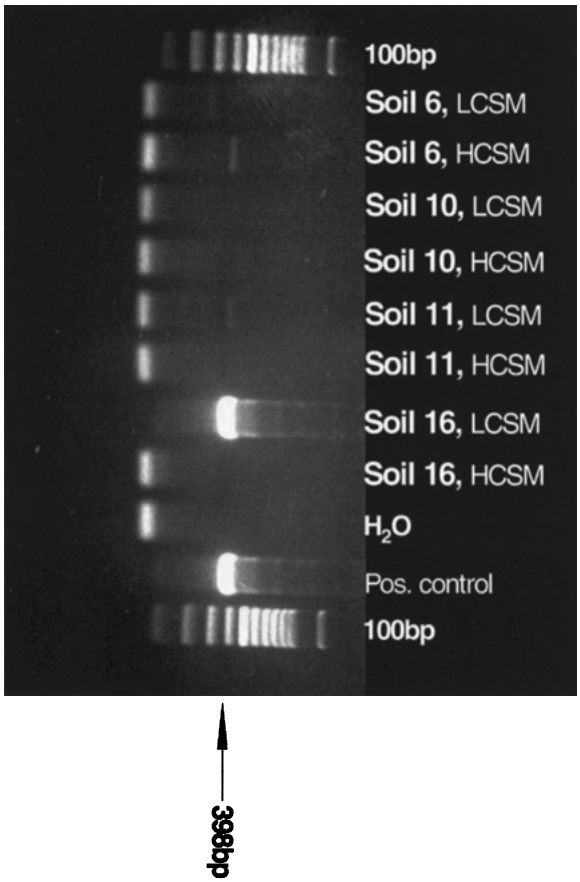

Figure 1. Amplification products from soil DNA extractions using main primers PBAW-10 and PBAW-11 and the nested primers PBTZS-3 and PBTZS-4. 20 µl of extracted DNA was used. Lanes are numbered from top to bottom. Lanes 2 and 3: soil 6; lanes 4 and 5: soil 10; lanes 6 and 7: soil 11; lanes 8 and 9: soil 16; lane 10: sterile distilled water; lane 11: extracts of roots infected with *P. brassicae* from field 7; lanes 1 and 12: molecular size marker (100 bp ladder). The soils were extracted with 4 g l^{-1} skim milk powder (LCSM) or with 40 g l^{-1} skim milk powder (HCSM).

equivalent to 26 µg to 2.6 pg DNA. The main primer pair PBAW-10 and PBAW-11 with nested primers PBTZS-3 and PBTZS-4 gave amplification products at a DNA content of 260 pg, while the primer pair PBAW-12 and PBAW-13 with nested primers PBTZS-3 and PBTZS-4 were less efficient (Table 4). The primers used by Faggian et al. (1999) gave amplification products at a DNA amount of 260 pg (Table 4).

Amplification of soil DNA extracts

With nested PCR (PBAW-10, PBAW-11 and PBTZS-3 and PBTZS-4) amplification products were only generated for sample 16 when extracted with a low concentration of skim milk (LCSM) (Figure 1). Extractions performed with the high concentration of skim milk (HCSM) did not generate amplification products. For the method of Faggian et al. (1999), both 20 µl and 1 µl of soil extract respectively were used in the PCR reactions. Using 20 µl amplification products were only generated with LCSM (Figure 2a), while using 1 µl amplification products were generated also with HCSM (Figure 2b).

Dilution series (10^{-1}, 10^{-2}, 10^{-3}) conducted with soils 16 and 11, were extracted with LCSM or HCSM and tested by Faggian's method. Three replicates of each dilution were amplified. In soil 16 visible products were received for all of the undiluted samples and for the samples diluted at 10^{-1} (Figure 3a) extracted with LCSM. The extract from soil 11 was more difficult to amplify, as only one of the undiluted samples, and one of the three 10^{-1} samples gave a visible product (Figure 3b). Using HCSM, results were more inconsistent. Soil 16 generated an amplification product in one of the undiluted samples and in one sample at 10^{-3}, while soil 11 generated an amplification product in one of the diluted samples, but not in the undiluted sample (data not shown here).

Sequencing

P. brassicae DNA from soils 2, 11 and 16 respectively was amplified according to Faggian et al. (1999) and sequenced with primers PbITS-6 and PbITS-7. The two sequences were combined in order to obtain most of the 507 bp fragment for these three isolates. An alignment of the sequences together with *P. brassicae* from the UK (Ward, accession no Y12831) is presented in Figure 4. The three Swedish isolates have identical sequences, but differ in a few bases from the isolate sequenced by Ward and Adams (1998).

Discussion

The results presented show that a 398 bp DNA (Ito et al., 1997) and a 507 bp DNA (Faggian et al., 1999) region can be amplified from Swedish populations of *P. brassicae*. The protocols described enabled detection

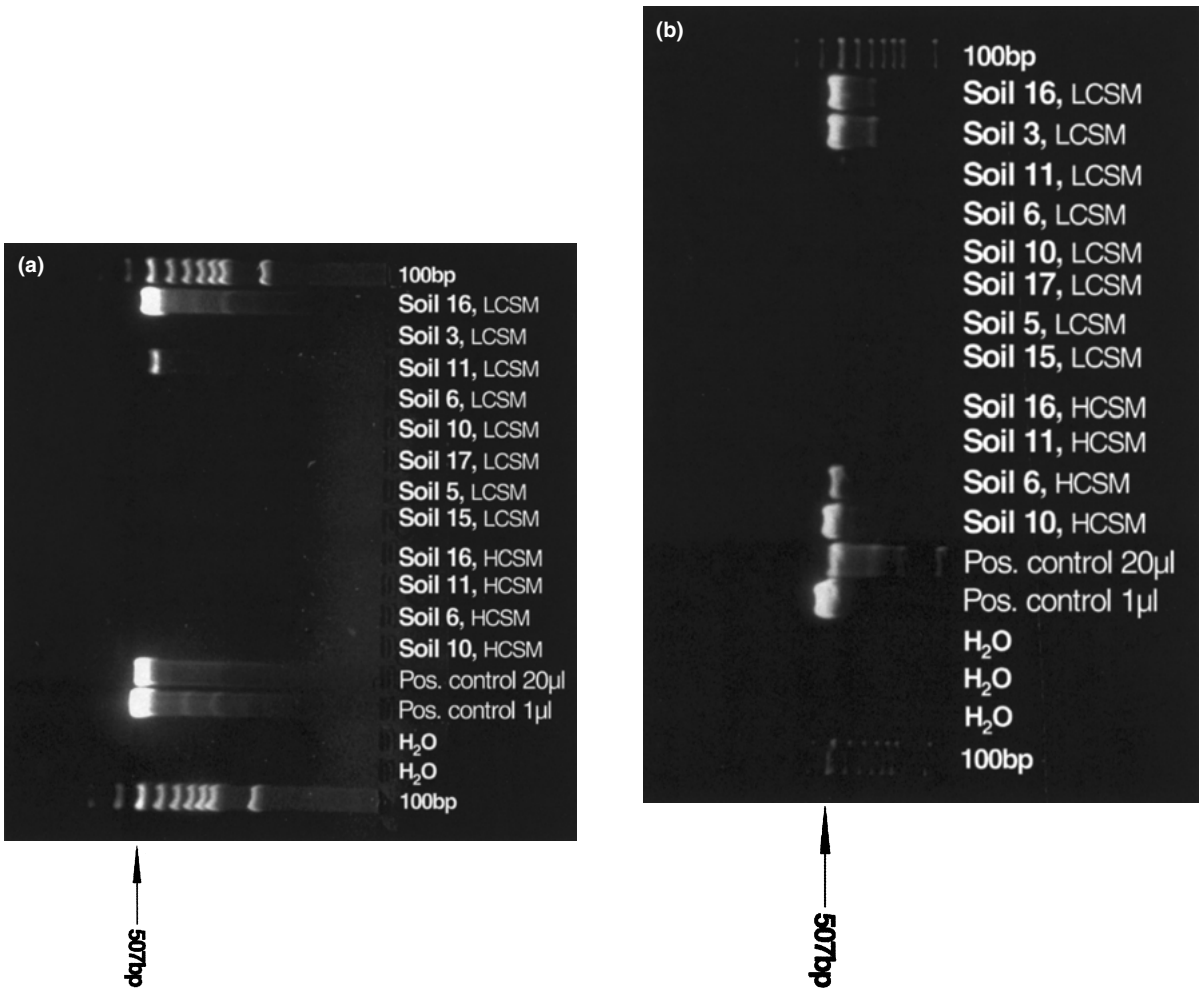

Figure 2. Amplification products from soil DNA extractions using main primers PbITS1 and PbITS2 and nested primers PbITS6 and PbITS7. (a) 20 µl of extracted DNA. Lanes are numbered from top to bottom. Lane 2: soil 16; lane 3: soil 3; lane 4: soil 11; lane 5: soil 6; lane 6: soil 10; lane 7: soil 17; lane 8: soil 5 (uninfested); lane 9: soil 15 (uninfested); lane 10: soil 16; lane 11: soil 11, lane 12: soil 6; lane13: soil 10; lane 14: extracts from roots infected with *P. brassicae* field No. 2; 20 µl; lane 15 positive control (same as lane 14) 1 µl; lanes 16 and 17: sterile distilled water; lanes 1 and 18 molecular size marker (100 bp ladder). The soils were extracted with 4 g l^{-1} skim milk powder (LCSM) or with 40 g l^{-1} skim milk powder (HCSM). (b) 1 µl of extracted DNA. Lanes 1–17 numbered as in (a); lane 18: sterile distilled water; lane 19: molecular size marker.

of DNA in naturally infested soils with an inoculum level of *P. brassicae* corresponding to a DSI higher than 21 (more than 35% infected plants in a bioassay). A soil infestation threshold level that may be used by growers is defined as a DSI of 10, corresponding to about 20% infected plants (Wallenhammar, 1999). *P. brassicae* was detetcted in different soil types, where the clay content ranged from 8 to 29%, and the humus content ranged from 0.4 to 11.9% (Table 1). However, it was not possible to reliably detect *P. brassicae* in the soil sample with the lowest infection level corresponding to a DSI of 8. Results furthermore indicate that the primers used are general for *P. brassicae*, and consequently the nested PCR assay has a potential to be developed as a routine detection method.

The application of nested PCR provided greater sensitivity compared to a single round PCR. When amplifying DNA extracted from infected roots, an amplification product was generated with primers (PBTZS-3 and PBTZS-4) with the template diluted

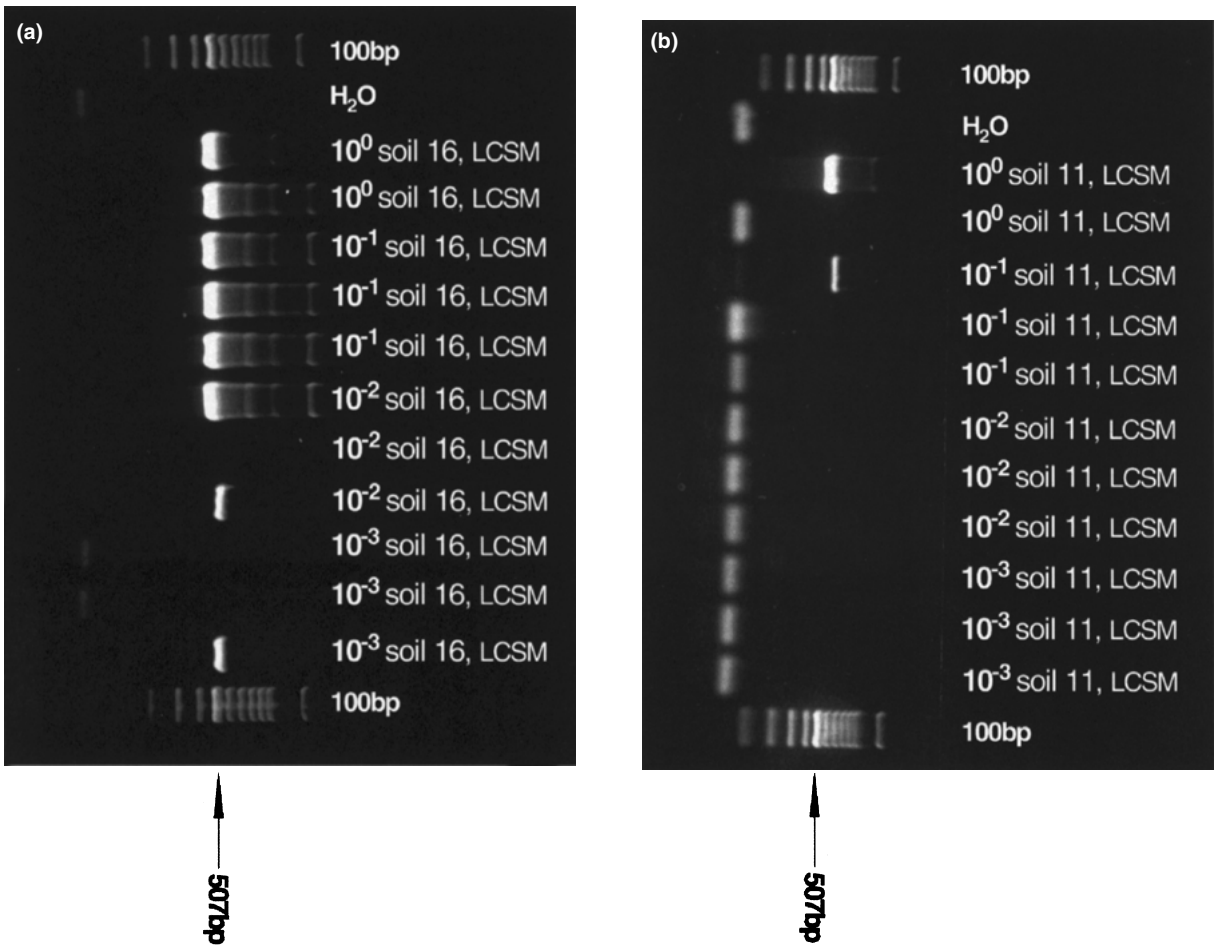

Figure 3. Amplification products from a dilution series (10^{-1}, 10^{-2}, 10^{-3}) of soil DNA extractions from two soils (a) Soil No. 16. (b) Soil No. 11. Three replicates of each dilution were amplified. Main primers, PbITS1 and PbITS2 and nested primers, PbITS6 and PbITS7. The soils were extracted with 4 g l^{-1} skim milk powder.

to 260 ng. Using the outer primers (PBAW-10 and PBAW-11) in a nested PCR, sensitivity increased to 260 pg (Table 4). In addition, diluting DNA often gave better PCR results, probably due to a lower amount of inhibiting substances in the reaction mix. This seems to be true for the humus-rich soil (No. 3) that resulted in an amplification product when 1 µl sample was used, but not when 20 µl was used (Figures 2a,b). Bulman and Marshall (1999) also reported amplification from humic acid contaminated samples with nested PCR. A drawback of dilution is that there then might be too little DNA for amplification, and for this reason soil No. 11 (LCSM) only gave a visible product when 20 µl was used as template but not when 1 µl was used. For routine testing both undiluted and diluted samples of the extracted DNA should be used.

P. brassicae DNA was detected in most of the soil samples by using the method of Faggian et al. (1999) (Figures 2a,b). We believe that the repetitive rDNA region in this case enables higher sensitivity compared to the single-copy target in our protocol. In both cases, a carrier skim milk was used according to Volossiouk et al. (1995) in order to minimize adsorption and degradation of DNA. The high level of skim milk powder suggested to improve detection in clay soil samples did not, however, improve the results in our studies (Table 3).

As in all soil testing, the collection and extraction of a representative soil sample is of primary importance. For commercial bioassays used for detecting *P. brassicae*, a soil sample of 4–6 kg is recommended (Clarkson and Brokenshire, 1984; Gröntoft, 1986;

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          1            15 16           30 31           45 46           60 61           75 76           90
1 Soil11  GCGCGCGCCATGCGA CACTGTTAAATTGCG GGGATCCCCTGAAGC TTTGTCCTACCAAGT GCTTGTGCGCGAAAG CGCCAGCATGGCCGA     90
2 Soil14  GCGCGCGCCATGCGA CACTGTTAAATTGCG GGGATCCCCTGAAGC TTTGTCCTACCAAGT GCTTGTGCGCGAAAG CGCCAGCATGGCCGA     90
3 Soil2   GCGCGCGCCATGCGA CACTGTTAAATTGCG GGGATCCCCTGAAGC TTTGTCCTACCAAGT GCTTGTGCGCGAAAG CGCCAGCATGGCCGA     90
4 Ward-98 GCGCGCGCCATGCGA CACTGTTAAATTGCG GGGACCCCCTGAAGC TTTGTCCTACCAAGT GCTTGTGCGCGAAAG CGCCAGCATGGCCGA     90
                                          *
          ---------------------- P b. insertion in 18 S ----------------------------------------------------

          91          105 106         120 121         135 136         150 151         165 166         180
1 Soil11  GCCAATCGCCCTGGG TATGGTAACAACGGA CAAGGATGTTATATA TGGGTGATCCGCAGC CAAGTCCTACGTCGA ATCGCGCATGTTCGA    180
2 Soil14  GCCAATCGCCCTGGG TATGGTAACAACGGA CAAGGATGTTATATA TGGGTGATCCGCAGC CAAGTCCTACGTCGA ATCGCGCATGTTCGA    180
3 Soil2   GCCAATCGCCCTGGG TATGGTAACAACGGA CAAGGATGTTATATA TGGGTGATCCGCAGC CAAGTCCTACGTCGA ATCGCGCATGTTCGA    180
4 Ward-98 GCCAATCGCCCTGGG TATGGTAACAACGGA CAAGGATGTTATATA TGGGTGATCCGCAGC CAAGTCCTACGTCGA ATCGCGCATGTTCGA    180

          ---------------------- P b. insertion in 18 S ----------------------><------------------------

          181         195 196         210 211         225 226         240 241         255 256         270
1 Soil11  TATGGAACGGGTTCA CAGACTAGATAGCAG TGGGTGGCACACT-- CGTGTGTCGCTTAAG ATATAGTCGGTCTCG TGCCGAAACGCACGG    268
2 Soil14  TATGGAACGGGTTCA CAGACTAGATAGCAG TGGGTGGCACACT-- CGTGTGTCGCTTAAG ATATAGTCGGTCTCG TGCCGAAACGCACGG    268
3 Soil2   TATGGAACGGGTTCA CAGACTAGATAGCAG TGGGTGGCACACT-- CGTGTGTCGCTTAAG ATATAGTCGGTCTCG TGCCGAAACGCACGG    268
4 Ward-98 TATGGAACGGGTTCA CAGACTAGATAGCAG TGGGTGGCACACACA CGTGTGTCGCTTAAG ATATAGTCGGTCTCG TGCCGAAACGCACGG    270
                                          ***
          ---------------------- P b. insertion in 18 S ----------------------------------------------------

          271         285 286         300 301         315 316         330 331         345 346         360
1 Soil11  GGAGGTGATCGACGC TCTTGCGTGTCGCTG TATTCGACAACTGCA AGTGTGCGTCACATG CACATACACACGTTC CGTAGGTGAACCTGC    358
2 Soil14  GGAGGTGATCGACGC TCTTGCGTGTCGCTG TATTCGACAACTGCA AGTGTGCGTCACATG CACATACACACGTTC CGTAGGTGAACCTGC    358
3 Soil2   GGAGGTGATCGACGC TCTTGCGTGTCGCTG TATTCGACAACTGCA AGTGTGCGTCACATG CACATACACACGTTC CGTAGGTGAACCTGC    358
4 Ward-98 GGAGGTGATCGACGC TCTTGCGTGTCGCTG TATTCGACAACTGCA AGTGTGCGTCACATG CACATAT--ACGTTC CGTAGGTGAACCTGC    358
                                                                                  ***
          ---------------------- P b. insertion in 18 S ----------------------------||---- 18S ---------

          361         375 376         390 391         405 406         420 421         435 436         450
1 Soil11  GGAAGGATCATTAAC ACAGTGGGCGGCCCT AGCGCTGCATCCCAT ACCCAACCCCATGTG AACCGGTGACGTGCG GCGACTCCAGCTGCG    448
2 Soil14  GGAAGGATCATTAAC ACAGTGGGCGGCCCT AGCGCTGCATCCCAT ACCCAACCCCATGTG AACCGGTGACGTGCG GCGACTCCAGCTGCG    448
3 Soil2   GGAAGGATCATTAAC ACAGTGGGCGGCCCT AGCGCTGCATCCCAT ACCCAACCCCATGTG AACCGGTGACGTGCG GCGACTCCAGCTGCG    448
4 Ward-98 GGAAGGATCATTAAC ACAGTGGGCGGCCCT AGCGCTGCATCCCAT ATCCAACCCCATGTG AACCGGTGACGTGCG GCGACTCCAGCTGCG    448
                                                        *
          ---- 18S ---||---------------------------------- ITS 1 ----------------------------------------

          451
1 Soil11  TGTTT    453
2 Soil14  TGTTT    453
3 Soil2   TGTTT    453
4 Ward-98 TGTTT    453

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Figure 4. Alignment of sequences of Swedish isolates of *Plasmodiophora brassicae* and a UK isolate Ward and Adams (1998) (accession no Y 12831). The sequence starts 27 bp downstream from the binding site of primer PbITS-6, and ends 28 bp upstream of primer PbITS-7's binding site. Nucleotides labelled * show differences between the Swedish and the UK isolates.

Wallenhammar, 1996). The size of the soil sample used for extraction of DNA often ranges from 50 to 1000 mg (Cullen et al., 1999; Ernst et al., 1996; Faggian et al., 1999; Ito et al., 1999; Elväng, 1998; Volossiouk et al., 1995). We did not detect amplification products consistently in some of the soil samples, although a high DSI (88) occurred in the corresponding soil bioassay. In these studies the size of our soil samples might have been be too small (250 mg) for detecting a soil-borne pathogen known to have a patchy nature of disease incidence (Wallenhammar, 1998). A larger soil sample should improve the yield of DNA from field soils, and may be necessary for detection in soils with low levels of inoculum, e.g., with a DSI $<$ 20. Also, DNA extraction from soil samples might require different extraction methods for different soil types. There are reports of various other soil DNA extraction and purification methods (Cullen et al., 1999; Faggian et al., 1999). We believe that a more efficient extraction method will facilitate DNA extraction from soil samples with a low level of inoculum.

In the 18S/ITS 1 regions sequenced from three Swedish *P. brassicae* isolates, we found complete identity, but this differed in a few bases from the UK isolate sequenced by Ward and Adams (1998). These sequence differences indicate variations in populations of *P. brassicae*, and may be useful information in studying the diversity among *P. brassicae* populations. However, in order to undertake more extensive population studies, e.g., to facilitate resistance breeding (Crute et al., 1980; Wallenhammar et al., 2000), more detailed and informative markers will be required.

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