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Rapid Detection of *Phytophthora infestans* by PCR

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ABSTRACT

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Late blight caused by the oomyceteous pathogen *Phytophthora infestans* is a devastating disease of potato and tomato worldwide. The objective of the present study is to develop a method for rapid detection of *P. infestans* from potato tubers and tomato seeds. Three oligonucleotide primers (Pi1S-1, Pi2A-1, and Pi2A-2) were designed based on the internal transcribed spacer sequences of the fungus, which made two primer sets: Pi1S-1/Pi2A-1 and Pi1S-1/Pi2A-2. PCR analysis demonstrated that DNA fragments of predicted size, using either Pi1S-1/Pi2A-1 or Pi1S-1/Pi2A-2 as the primer set, were amplified from all the *P. infestans* isolates tested. Moreover, when the amount of template DNA was reduced to 1 pg, PCR primed with Pi1S-1/Pi2A-2 could still give rise to expected DNA fragment. No signals were detected with DNA from other fungal species analyzed in the study. To detect *P. infestans* present in the potato tuber, a rapid method for DNA extraction followed by PCR with Pi1S-1/Pi2A-2 was established. This method will provide a useful tool for rapid detection of *P. infestans* in potatoes and tomatoes.

Key words: Internal transcribed spacer (ITS), PCR detection, *Phytophthora infestans*.

INTRODUCTION

Phytophthora infestans (Mont.) de Bary is an important pathogen of potato and tomato worldwide [20]. Severe late blight epidemics were reported on both potatoes and tomatoes in Europe and America [7,8] in recent years. In Taiwan, *P. infestans* has been known to exist since the early 1900s [12,19]. However, it was not until 1997 when outbreak of serious potato late blight was first noticed in the Houli area of Taichung [3]. Since then, the late blight disease has become a significant threat to potato production throughout Taiwan. The mating type of *P. infestans* isolated from the diseased plants thus far were all A1 [1,2,3,9]. The pathogen survives in the infected tubers and tomato seeds [1]. Once planted in the field, they will provide the primary inoculum for epidemics if weather conditions are favorable, and result in severe losses due to foliar destruction and rotting of blighted tubers.

Traditional methods for detection of *P. infestans* involve eye inspection, examination under microscopy, and isolation of the fungus from the plant tissues. Isolation of *P. infestans* can be difficult due to poor growth of fungal mycelia on general media. Moreover, the infected buds or potato tubers may appear symptomless. A rapid and sensitive method for detecting *P. infestans* is thus needed to improve the process of pathogen identification and to prevent the introduction of infected materials into fields. A variety of molecular tools have been used to differentiate *Phytophthora* spp., including blot hybridization [15], polymerase chain reaction [6,10,12,17,23], and single-stranded conformation polymorphism [21]. ITS-based PCR primers developed for detection of *P. infestans*, however, were found to amplify DNA also from *P. colocasiae* [22] or *P. cactorum* [23]. The objective of this study was to design oligonucleotides based on the internal transcribed spacer (ITS) of the ribosomal RNA genes and assayed for their specificity as *P. infestans*-specific PCR primers. A simple process for quick extraction of DNA from the potato tuber followed by PCR analysis, using Pi1S-1 and Pi2A-2 as primers, was also established. This technique will provide a valuable tool for specific detection of *P. infestans* in infected potato and tomato.

Materials and Methods

Fungal cultures and growth conditions

Fungal isolates used in the study were listed in Table 1. Isolates of *Phytophthora* spp., *Pythium splendens*, and *Peronophythora litchii* were from the third author, *Pythium*

Table 1. Fungal isolates analyzed in the study

Speciesc	Isolate	Location	Host
Phytophthora infestans	92251	Shinyi, Nantow	*Lycopersicum esculentum* (tomato) 番茄
P. infestans	95039	Mayfeng, Nantow	*Solanum tuberosum* (potato) 馬鈴薯
P. infestans	97003	Chiayi	*L. esculentum*
P. infestans	98005	Houli, Taichung	*S. tuberosum*
P. infestans	98029	Dounan, Yunlin	*S. tuberosum*
P. infestans	98045	Shikou, Chiayi	*S. tuberosum*
P. infestans	98057	Tsautuen, Nantow	*L. esculentum*
P. infestans	98062	Wufeng, Taichung	*L. esculentum*
P. infestans	98087	Sanyi, Miaoli	*S. tuberosum*
P. infestans	98102	Yuanshan, Ilan	*L. esculentum*
P. infestans	99001	Tsautuen, Nantow	*L. esculentum*
P. infestans	99005	Guoshing, Nantow	*L. esculentum*
P. infestans	99013	Houli, Taichung	*L. esculentum*
P. infestans	99017	Houli, Taichung	*S. tuberosum*
P. infestans	99022	Taibau, Chiayi	*L. esculentum*
P. infestans	99034	Guanshan, Taitung	*L. esculentum*
P. infestans	99037	Luye, Taitung	*L. esculentum*
P. infestans	99063	Chian, Hualien	*L. esculentum*
P. infestans	20001	Douliou, Yunlin	*S. tuberosum*
P. infestans	20005	Dounan, Yunlin	*S. tuberosum*
P. infestans	20032	Luye, Taitung	*L. esculentum*
P. infestans	20040	Rueiyen, Hawlian	*L. esculentum*
P. infestans	20056	Shueili, Nantow	*L. esculentum*
P. cactorum		Taiwan	*Fragaria chiloensis* (straw berry) 草莓
P. capsici	97023	Wufeng, Taichung	*Capsicum annuum* (pepper) 辣椒
P. cinnamomi	94006	Chiayi city	*Persea americana* (Avocado) 酪梨
P. citricola	98131	Chienjien, Nantow	*Pleione formosana* (Taiwan pleione) 一葉蘭
P. citrophthora	95004	Jiaushi, Ilan	*Fortunella sp*. (kumquant) 金柑
P. colocasiae	91077	Minshiung, Chiayi	*Colocasie esculenta* (taro) 芋
P. cryptogea	90127	Yungching, Changhua	*Euphobia pulcherrima* (poinsettia) 聖誕紅
P. drechsleri	90155	Changhua	*Curcuma domestica* (curcuma) 葛鬱金
P. melonies	98107	Tainan	*Momordica charantia* (bitter gourd) 苦瓜
P. meadii	PMo8	Taipei	*Zantedeschia aethiopica* (white arum lily) 白色海芋
P. palmivora	9253	Taitung	*Phalaenopsis* sp. 蔓綠絨
P. parasitica	771-1	United States	*Citrus* sp. 柑橘
P. parasitica	991-3	United States	*Citrus* sp.
P. parasitica	95023	Wufeng, Taichung	*Eriobotrya japonica* (loquat) 枇杷
P. parasitica	96085	Yungching, Changhua	*Eriobotrya japonica*
P. parasitica	93122	Fanlu, Chiayi	*Nicotiana tabaccum* (tobacco) 煙草
P. parasitica	98161	Wufeng, Taichung	*Adenium obesum* (Desert rose) 沙漠玫瑰
P. parasitica	9241	Taitung	*Dieffenbachia maculata* (dieffenbachia) 黛粉葉
P. parasitica	92033	Taitung	*Saintpulia ionantha* (African violet) 非洲堇
Fusarium solani	32446		
F. moniliforme	32878		
Nimbya gomphrenae		Taipei	*Gomphrena globosa* (globe amaranth) 千日紅
Phoma nelumbii	CCRC35162	Tatung, Ilan	*Colocasie esculenta* (taro) 芋
Rhizoctonia solani AG 2-1	PS-4	Tokushima, Japan	*Pisum sativum* (pea) 豌豆
R. solani AG 5	GM-10	Nagano, Japan	*Glycine max* (soybean) 大豆
R. solani AG 9	ATCC-62804		
Peronophythora litchii	97029	Shueili, Nantow	Litchi chinensis (litchi) 荔枝
Pythium aphanidermatum	PY1464	Taipei	Cucumis sativus (cucumber) 胡瓜
Py. splendens	Pys10	Puli, Nantow	Piper betle (betle pepper) 荖花

aphanidermatum (PY1464) was provided by professor Huann-Ju Sheih (Dept. Plant Pathology, National Taiwan University), and other fungal isolates were from professor Shean-Shong Tzean (Dept. Plant Pathology, National Taiwan University). All fungi were maintained on agar plates and subcultured once per month. Rye B medium [4] and 5% V8 agar (5% Campbell's V-8 juice, 0.02% $CaCO_3$ and 2% Bacto agar) were used for cultivation of *P. infestans* and other fungi, respectively.

Liquid media were used to obtain mycelia for DNA analysis. Five culture blocks (approximately 6 x 6 x 6 mm) from each fungus were transferred into a 500-ml flask containing 100 ml of culture medium. *P. infestans* was grown in Rye A medium [4] at 20℃ for 20 days, other *Phytophthora* spp. in 5% V8 juice (5% Campbell's V-8 juice and 0.02% $CaCO_3$) at 25℃ for 10 days, and *Py. aphanidermatum* in 10% V8 juice (10% Campbell's V-8 juice and 0.02% $CaCO_3$) at 30 ℃ in the dark for 5 days. Other fungi were grown in potato dextrose broth (Difco Laboratories, Detroit, Michigan) for 8 days. Mycelia were harvested by filtration and frozen at -80℃ until use.

Isolation of fungal DNA

Fungal DNA was isolated from mycelia by the cetyltrimethylammonium bromide (CTAB) method of Sambrook *et al.* [18] with some modifications. Following lyophilization, mycelia (1 g) were ground with mortar and pestle in the presence of liquid nitrogen and subsequently resuspended in 10 ml of DNA extraction buffer [containing 1% CTAB, 0.7 M NaCl, 25 mM EDTA, 50 mM Tris-Cl (pH 8.0) and 1% β-mercaptoethanol]. The mixture was incubated at 65℃ for 1 hour and then subjected to phenol-chloroform extraction. Afterwards, the upper phase was collected, mixed with 0.6 volume of isopropanol, and incubated at -20℃ overnight. Pellet obtained in the next day was resuspended in TE buffer (0.1 mM EDTA, 10 mM Tris, pH 8.0) and used as the template for PCR. Concentration of DNA was determined with spectrophotometry (GeneQunat II, Pharmacia Biotech).

Oligonucleotide primer design and polymerase chain reaction

PCR primers for amplification of *P. infestans* DNA were designed based on multiple sequence alignments obtained with PILEUP [5], which included internal transcribed spacer sequences of several *Phytophthora* spp. The upstream primer (Pi1S-1) was designed based on the ITS1 sequence (5'-GCGTT GGGAC TCCGG TCTGA GC-3'), while two downstream primers were designed based on the ITS2 sequence: Pi2A-1 (5'-CGCAA GACAC TTCAC ATCTG GG-3') and Pi2A-2 (5'-CGCCA CAGGA GGAAA ATCAC-3'). PCR was performed using either Pi1S-1/Pi2A-1 or Pi1S-1/Pi2A-2 as the primer set. Each reaction (20 μl) consisted of 10 ng of fungal DNA, 1.25 μM of oligonucleotide primers, 0.2 mM dNTP, 1X PCR buffer, and 1 U of DyNazyme™ II DNA polymerase (Finnzymes). Amplification was initiated by denaturation at 94℃ for 5 min, followed by 25 cycles of [95℃/30 sec-65℃/30 sec-72℃/45 sec] and a 10-min extension at 72℃ in a thermocycler (GeneAmp PCR System 2400, Perkin elmer). PCR amplified products were analyzed by 1.5% agarose gel electrophoresis in 1x TAE.

PCR detection of *P. infestans* from diseased potato tubers

Potato tubers were inoculated with mycelia of *P. infestans* isolates and incubated at 20℃ for the appearance of symptoms. For detection of *P. infestans* by PCR, slices were excised from the tuber and crude DNA was isolated according to the method developed by Wang *et al.* [24] and modified by Tooley *et al* [22]. After addition of 0.5 N NaOH, potato tuber slices were macerated in the microcentrifuge tube and subjected to centrifugation at 17,000 g for 5 min. The supernatant was collected and mixed with 9 volume of 0.1 M Tris buffer (pH 8.0). Aliquot of the mixture was then used as the template for PCR, which was performed as described in the previous section.

Results

Primer design and specificity

In order to design the oligonucleotide primers for PCR, ITS1 and ITS2 sequences of multiple *Phytophthora* spp. were collected from Genebank and aligned by PILEUP [5] (data not shown). One upstream primer (Pi1S-1) and two downstream primers (Pi2A-1 and Pi2A-2) were designed based on the ITS1 and ITS2 region of *P. infestans*, respectively, which deviates most significantly from other species. Amplification of *P. infestans* DNA using Pi1S-1 and Pi2A-1 as the primer set would generate a PCR product of approximately 350 bp. PCR with Pi1S-1/Pi2A-2, on the other hand, would give rise to an amplified product of 550 bp in length. To evaluate the specificity of the primer, PCR was performed using Pi1S-1/Pi2A-1 or Pi1S-1/Pi2A-2 as the primer set, and DNA from 13 fungal species as the template: *P. capsici*, *P. cinnamomi*, *P. citrophthora*, *P. cactorum*, *P. colocasiae*, *P. infestans* (isolate number 20001), *P. palmivora*, *P. parasitica*, *P. citricola*, *P. cryptogea*, *P. drechsleri*, *P. meadii*, and *Peronophythora litchii*. Analysis of the amplified products by agarose gel electrophoresis demonstrated that PCR with Pi1S-1/Pi2A-1 gave rise to amplified products of approximately 350 bp from *P. infestans* as well as other *Phytophthora* spp. (*P. capsici*, *P. citrophthora*, and *P. colocasiae*) (Fig. 1a). PCR with Pi1S-1/Pi2A-2, on the contrary, generated a DNA band with expected size only from *P. infestans* (Fig. 1b), but not other fungal species tested. To further evaluate the specificity of Pi1S-1/Pi2A-2, experiments were performed with more fungal isolates, including 23 isolates of *P. infestans*, 8 isolates of *P. parasitica*, *Fusarium solani*, *F. moniliforme*, *Nimbya*

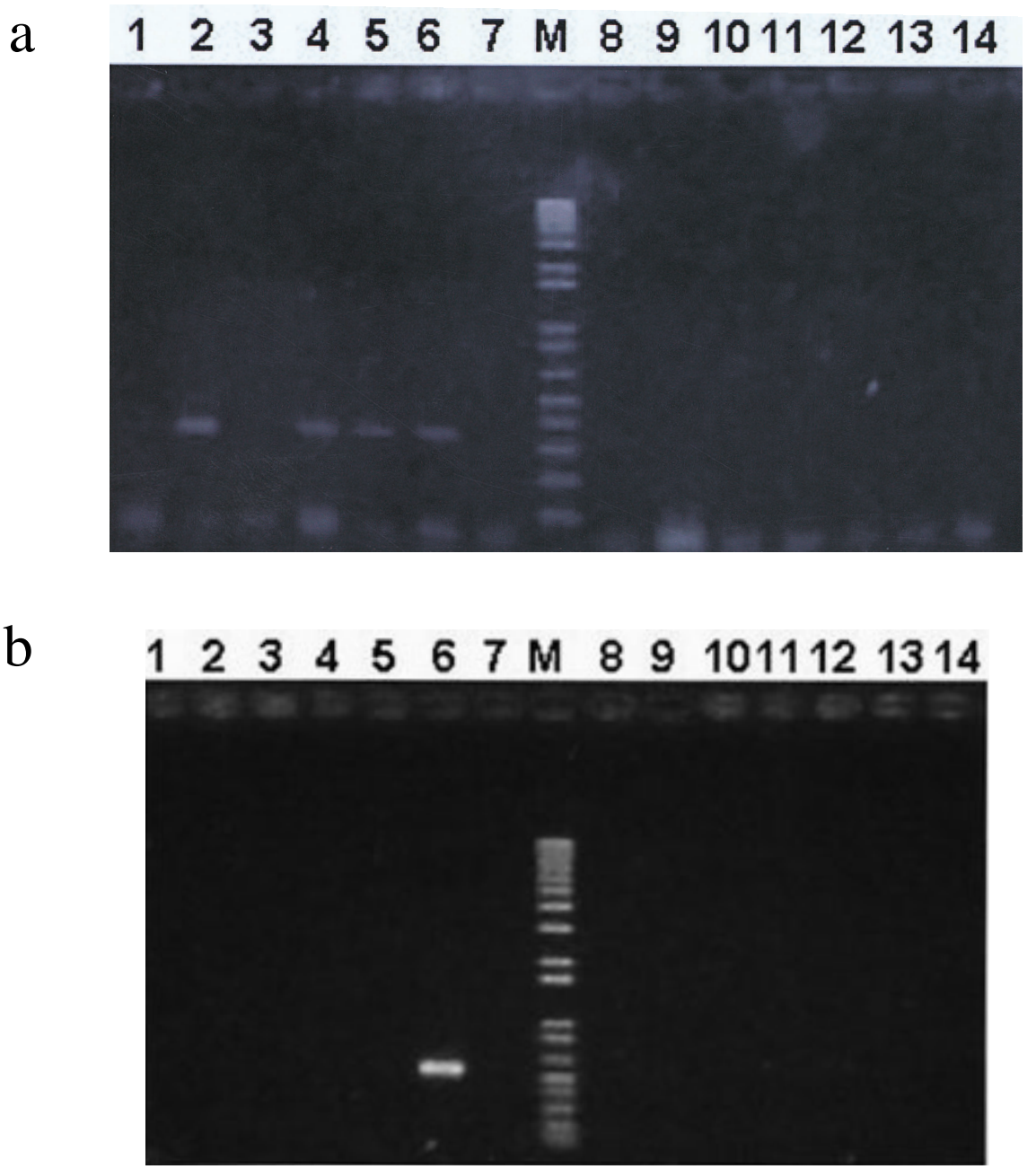


Fig. 1. PCR amplification of DNA prepared from *Phytophthora* spp. and *Peronophythora litchii*. PCR was performed using Pi1S-1/Pi2A-1 (panel a) or Pi1S-1/Pi2A-2 (panel b) as the primer set. The amplified products were analyzed by 1.5% agarose gel electrophoresis. Fungal isolates analyzed include: *P. parasitica* (lane 1), *P. capsici* (lane 2), *P. cinnamomi* (lane 3), *P. citrophthora* (lane 4), *P. colocasiae* (lane 5), *P. infestans* (isolate number 20001) (lane 6), *P. palmivora* (lane 7), *P. citricola* (lane 8), *P. cryptogea* (lane 9), *P. drechsleri* (90155, lane 10), *P. drechsleri* (lane 11), *P. meadii* (lane 12), *P. cactorum* (lane 13), and *Peronophythora litchii* (lane 14). M: 1 kb DNA ladder (BRL).

gomphrenae, *Phoma nelumbii*, *Rhizoctonia solani* (AG 2-1, AG 5, and AG 9), *Pythium aphanidermatum*, and *Py. splendens* (Table 1). Analysis of the PCR products by agarose gel electrophoresis indicated that the 550-bp fragment appeared with every *P. infestans* isolate being analyzed (Fig. 2). No amplification of DNA occurred, however, from all other fungal species (data not shown). These results demonstrated that, of all the fungal species being analyzed, PCR with Pi1S-1/Pi2A-2 amplified the 550-bp fragment only from *P. infestans* DNA. Thus, they were used in the following experiments.

Analysis of PCR sensitivity

To determine the minimal amount of template DNA that is enough for generation of the amplified product, PCR was performed using Pi1S-1/Pi2A-2 as the primers and different

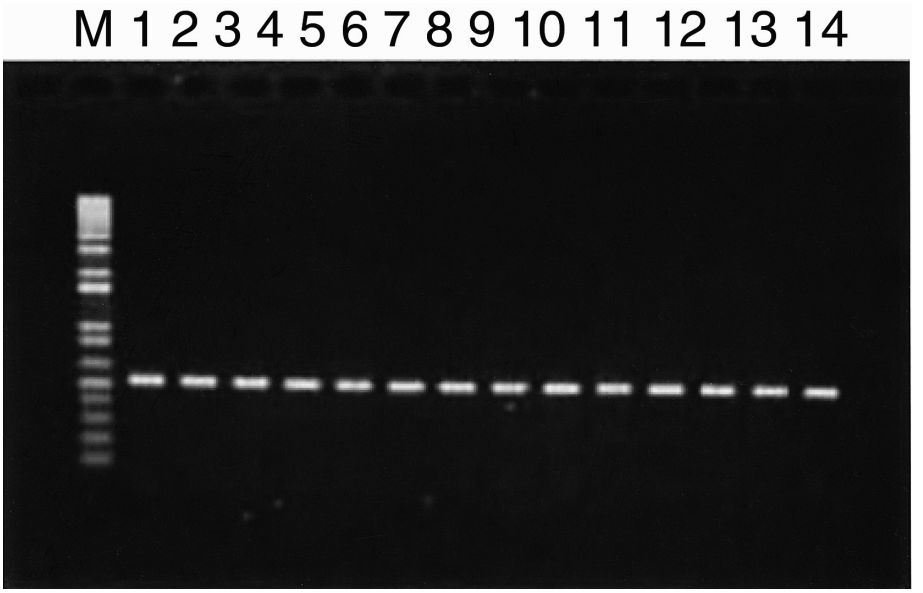


Fig. 2. PCR amplification of DNA prepared from 14 representative isolates of *Phytophthora infestans*. PCR was performed using Pi1S-1/Pi2A-2 as the primer set. The amplified products were analyzed by 1.5% agarose gel electrophoresis. M: 1 kb DNA ladder (BRL).

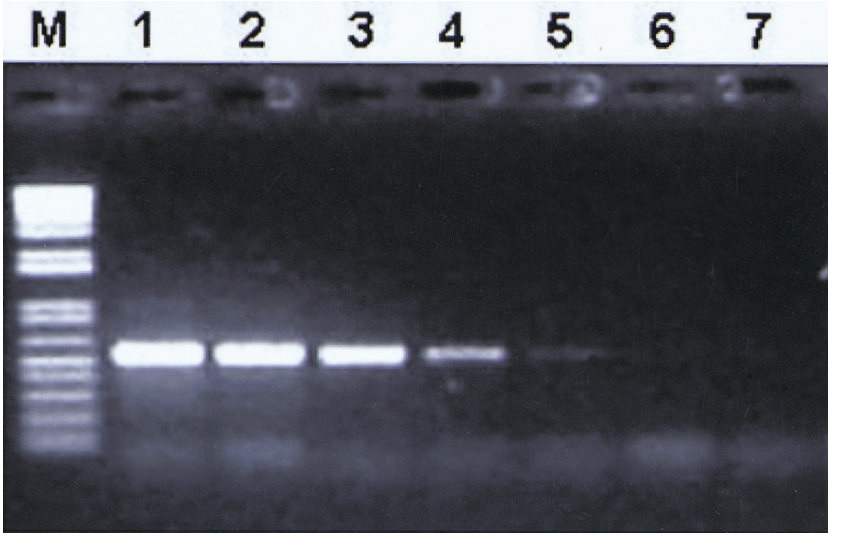


Fig. 3. Sensitivity analysis of the PCR detection method. PCR was performed using Pi1S-1/Pi2A-2 as the primer set, and indicated amount of *Phytophthora infestans* DNA as the template: 1 ng (lane 1), 100 pg (lane 2), 10 pg (lane 3), 1 pg (lane 4), 100 fg (lanes 5), and 10 fg (lane 6), 1 fg (lane 7). M: 1 kb DNA ladder (BRL).

amount of *P. infestans* DNA as the template. As shown in Fig. 3, PCR-amplified product from 1 pg of *P. infestans* DNA was detectable by ethidium bromide staining of the agarose gel.

Detection of *P. infestans* in infected potato tubers by PCR

To develop a PCR method for rapid detection of *P. infestans* in potato tubers, sample tissues were collected from tuber lesions, margins around the lesion, or symptomless areas at various distance from the tuber lesions, following inoculation of the potato tubers with *P. infestans*. Crude extract was prepared from the sample tissues by the NaOH method and analyzed by PCR primed with Pi1S-1/Pi2A-2. As shown in Fig. 4, PCR with Pi1S-1 and Pi2A-2 generated amplified products of expected size with samples collected from tuber lesions (lanes 1-3) and margins around the lesion (lanes 4 and 5), the same as the positive control (lane 8), which used gnomic DNA of *P. infestans* as the template for PCR. Two of the symptomless samples collected 1 cm away

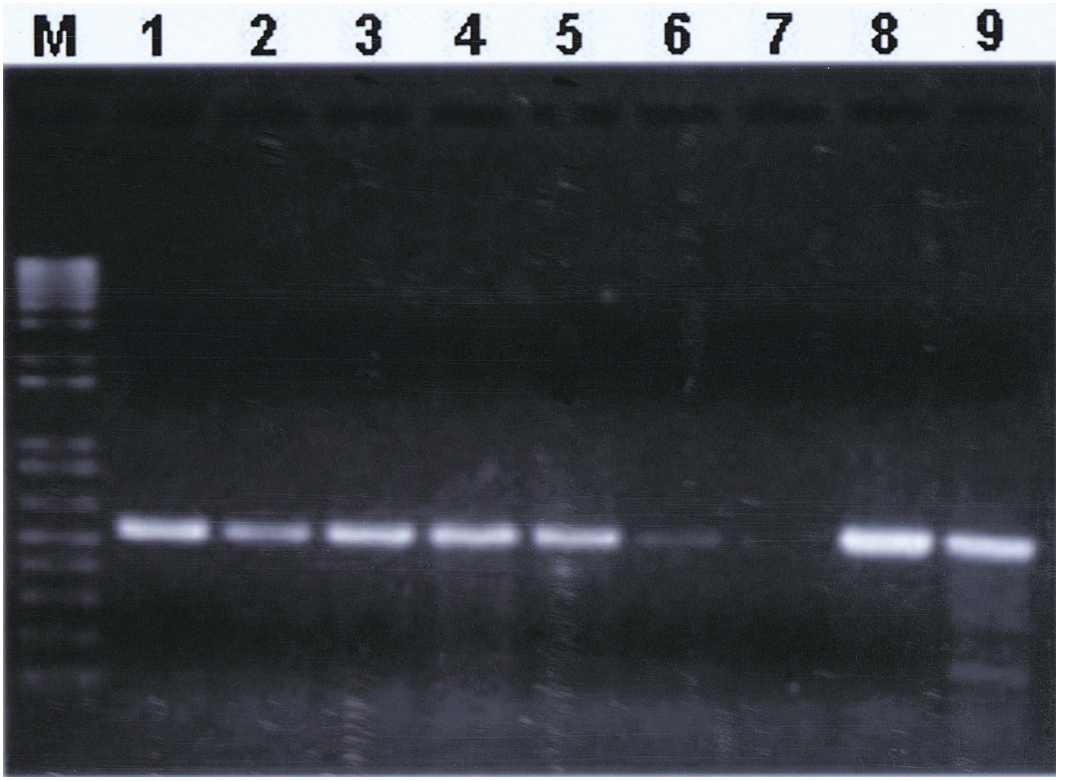


Fig. 4. PCR detection of *Phytophthora infestans* in potato tubers. Samples from infected tubers were process by the NaOH method and analyzed by PCR using Pi1S-1/Pi2A-2 as the primer set. DNA from potato tuber lesions (lanes 1-3), DNA from margins around the lesions (lanes 4 and 5), DNA from potato tissues 1 cm away from the lesions (lanes 6, 7, and 9), a positive control of PCR, which used genomic DNA of *P. infestans* as the template (lane 8). M: 1 kb DNA ladder (BRL).

from the lesions also gave rise to amplified products (lanes 6 and 9), although DNA band observed in lane 6 was barely visible. No amplification occurred with DNA from one of the symptomless samples (lane 7) and the uninoculated potato (data not shown).

Discussion

Recently, a variety of molecular tools have been used to differentiate *Phytophthora* spp., including blot hybridization [14], polymerase chain reaction [6,10,17,23], and single-stranded conformation polymorphism [21]. Compared to other techniques, detection of fungal pathogens by PCR is rapid, sensitive, and feasible for simultaneous detection of a second target by multiplex PCR [16,25,27]. In the present study, a PCR method for identification of *P. infestans* was developed, using primers (Pi1S-1, Pi2A-1, and Pi2A-2) designed according to the ITS sequences of the ribosomal RNA genes. ITS was chosen due to its high copy number in the genome of *P. infestans* [11,26], which allows detection with better sensitivity. Besides, ITS sequences of many *Phytophthora* spp. have been cloned and analyzed [14]. Analyses of these sequences by multiple sequence alignment would provide invaluable information for designation of species-specific PCR primers.

Preliminary test by PCR with annealing temperature of 60℃ indicated that both Pi1S-1/ Pi2A-1 and Pi1S-1/Pi2A-2 gave rise to amplified product with DNA from *P. infestans* as well as other *Phytophthora* spp. (data not shown). To improve the condition, the annealing temperature was raised to 65℃. This value was higher than the theoretical annealing temperature calculated from the primers. As demonstrated in Fig. 1, PCR with Pi1S-1 and Pi2A-2 yielded a strong PCR band with *P. infestans*, but not other fungal pathogens tested. PCR with Pi1S-1 and Pi2A-1, on the contrary, generated fragments of expected size with DNA from *P. capsici*, *P. citrophthora*, and *P. colocasiae*, in addition to *P. infestans*. To confirm its specificity, Pi1S-1/Pi2A-2 was further analyzed with DNA from 22 isolates of *P. infestans* and 8 isolates of *P. parasitica*, which is known to cause pink rot in potato. The result indicated that the amplified product was observed solely from DNA of *P. infestans*, but not *P. parasitica*. Thus, when the annealing temperature of PCR is set at 65℃, Pi1S-1/Pi2A-2 displays high specificity for *P. infestans*. Sensitivity analysis using serial dilutions of DNA as templates, on the other hand, demonstrated that PCR with Pi1S-1/Pi2A-2 could amplify DNA with a sensitivity limit of 1 pg. Previously, a couple of ITS-based PCR methods have been developed for detection of *P. infestans*. Oligonucleotide primers used in these studies, however, were found to amplify DNA also from *P. colocasiae* [22] or *P. cactorum* [23]. In addition, sensitivity of

the PCR assay was limited to 1-10 pg of DNA [22]. The PCR method developed in the present study, using Pi1S-1 and Pi2A-2 as the primers, is thus more specific and sensitive than other ITS-based assays which have been reported [22,23].

To establish a method for rapid detection of *P. infestans* which might exist in the potato seedlot, crude DNA was prepared from the potato tuber by the NaOH hydrolysis method, which is quick and easy to handle. As demonstrated in Fig. 4, PCR with Pi1S-1/Pi2A-2 could amplify DNA from potato tubers that have been inoculated with *P. infestans*. Amplified products were detected with DNA from tissues with obvious symptom as well as those without any symptom. No signal was detected, however, with DNA from healthy, uninoculated potatoes. Thus, the PCR method developed in this study is useful for detection of *P. infestans* which is present in the potato tubers, even in the absence of any visible lesions. However, for efficient application of this method in quarantine, more effort is required to explore the method of sampling due to large sample size of potato seedlot.

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摘 要

簡明宏[1]、黃麗君[1]、安寶貞[2]、劉瑞芬[1,3]. 2002. 晚疫病菌 PCR 檢測方法之建立. 植病會刊 43:25-32.
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晚疫病菌 *Phytophthora infestans* (Montagne) de Bary 是引起馬鈴薯與蕃茄晚疫病的病原眞菌，分佈遍及全世界，且造成非常嚴重之危害。爲了開發快速簡易之晚疫病菌檢測技術，以便應用於健康種薯檢測，我們根據疫病菌 (*Phytophthora* spp.) 核糖體核酸 ITS 序列之並列分析結果設計核酸引子 (Pi1S-1, Pi2A-1 與 Pi2A-2)，並以 Pi1S-1/Pi2A-1 或 Pi1S-1/Pi2A-2 爲引子對進行 PCR。分析結果顯示，不論以 Pi1S-1/Pi2A-1 或 Pi1S-1/Pi2A-2 爲引子對都可自所測試之晚疫病菌菌株增殖出預期大小之核酸片段，但自其他種類之供試菌株則觀察不到增殖產物，顯示其對晚疫病菌具有非常高之專一性。研究也顯示，將 DNA 減量至 1 pg 時，以 Pi1s-1/Pi2A-2 爲引子對仍可自晚疫病菌增殖出預期之核酸片段。因此，這一項技術應可成爲進行晚疫病菌鑑定與檢測之有用工具。此外，我們還建立了自接種馬鈴薯快速抽取 DNA，並以 Pi1s-1/Pi2A-2 爲引子對進行晚疫病菌檢測之技術，可應用於輔助馬鈴薯健康種薯之檢測。

關鍵詞：晚疫病菌、快速檢測、ITS、PCR