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Detection of *Phytophthora erythroseptica* in Soil Using Nightshade as Bait Combined with PCR Techniques

U. N. Nanayakkara · Mathuresh Singh · K. I. Al-Mughrabi · R. D. Peters

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Abstract Pink rot caused by *Phytophthora erythroseptica* is found in most major potato-growing regions of the world. The pathogen can survive for many years in soil by means of oospores which are disseminated from diseased potato tissues. The ability to detect the pathogen in soil could be a valuable management tool that enables growers to plan control strategies depending on the presence of pathogen propagules in a particular field. However, soils are one of the most challenging environmental matrices to obtain microbial DNA that will support PCR. A method was developed that combined traditional baiting technique with PCR methods to detect *P. erythroseptica* in infested soil samples. Hairy nightshade (*Solanum sarrachoides* Sendt.) and bitter nightshade (*Solanum dulcamara* L.) two leaf stage (TLS) seedlings and cotyledon leaves successfully baited *P. erythroseptica* from zoospore suspensions, artificially inoculated soils and naturally infested soils. The pathogen was detected in the bait tissue with PCR methods. PCR increased the precision of the bait test. However, time was still required for the pathogen to infect and develop on the bait tissues. Although *P. erythroseptica* was detected from some bait plants only after 2 days of incubation, 10 days of incubation produced consistent results across the replicates with hairy and bitter nightshade cotyledon leaves and TLS seedlings.

Resumen La pudrición rosada, causada por *Phytophthora erythroseptica,* se encuentra en la mayoría de las principales regiones productoras de papa en el mundo. El patógeno puede sobrevivir en el suelo por muchos años mediante oosporas que se diseminan de tejidos de papa enfermos. La habilidad para detectar al patógeno en el suelo podría ser una herramienta de manejo valiosa que permita a los productores planear estrategias de control dependiendo de la presencia de propágulos del patógeno en un campo en particular. No obstante, los suelos son de las matrices ambientales más retadoras para obtener DNA microbiano que pudiera soportar PCR. Se desarrolló un método que combinó la técnica de trampa tradicional con PCR para detectar *P. erythroseptica* en muestras de suelo infestado. Plántulas en estado de dos hojas (EDH) y las hojas cotiledonares de un tipo de hierba mora (*Solanum sarrachoides* Sendt.) y la dulcamara o mora trepadora (*Solanum dulcamara* L.), atraparon con éxito a *P. erythroseptica* de suspensiones de zoosporas, de suelos inoculados artificialmente y de suelos naturalmente infestados. Se detectó al patógeno en tejido trampa con métodos de PCR. La PCR aumentó la precisión de la prueba de la trampa. Sin embargo, aún se requirió de tiempo para que el patógeno infectara y se desarrollara en los tejidos trampa. Aunque se detectó a *P. erythroseptica* de algunas plantas trampa con solo dos días de incubación, 10 días de incubación produjeron resultados consistentes entre las repeticiones con hojas cotiledonares de hierba mora y la mora trepadora y de plántulas de EDH

Keywords Bitter nightshade · Hairy nightshade · Pink rot

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Introduction

Pink rot, caused by *Phytophthora erythroseptica*, is a disease of significant importance in many potato (*Solanum tuberosum* L.) growing regions of the world (Vargas and Nielsen 1972). The disease causes serious losses in the field and potato storage facilities. Pink rot develops in plants grown in soils approaching saturation from poor drainage, excessive precipitation or irrigation. Oospores of *P. erythroseptica* can survive for many years in soil (Vujičić and Park 1964), which are disseminated by diseased potato tissues. All infectious units of the pathogen including zoospores, sporangia and oospores may be found in soil (Lonsdale et al. 1980; Pethybridge 1913; Pethybridge 1914; Salas et al. 1997). Most commercially grown cultivars in Canada and the United States are susceptible to pink rot and breeding efforts to develop cultivars resistant to *P. erythroseptica* have been minimal (Peters et al. 2004). Although successful control of the pathogen can be achieved through applications of metalaxyl, metalaxyl-resistant strains of *P. erythroseptica* have been isolated in several potato-growing regions (Goodwin and McGrath 1995; Lambert and Salas 1994; Salas et al. 1998). Crop rotation with at least one alternate crop, such as oat, barley or soybeans, between potato crops has been shown to significantly reduce disease incidence (Lambert et al. 2001). Cultural practices such as minimizing tuber damage during harvest and grading out diseased tubers prior to storage reduce storage rot.

PCR analysis provides a sensitive and specific means to detect microorganisms in soil samples. However, successful detection requires efficient extraction and adequate purification from the co extracted contaminants such as humic and fulvic acids that are inhibitory to *Taq* DNA polymerase (Tebbe and Vahjen 1993; Tsai and Olson 1992). Soils are therefore one of the most challenging environmental matrices to obtain microbial DNA that will support PCR (Kuske et al. 1998). PCR assays have not been utilized to this date to detect *P. erythroseptica* infectious units from soil. Baiting with different plant tissue types that are susceptible to infection by the pathogen's zoospores is the most common way to detect and isolate *Phytophthora* species from infested soil samples (Erwin and Ribeiro 1996). However, subsequent isolation of *Phytophthora* from infected bait tissues requires time, special techniques, selective agar media, and considerable knowledge of the genus. Furthermore, infection and colonization of bait tissue by fast growing fungi such as *Pythium* can make isolation and identification of *Phytophthora* difficult or even impossible (Tsao 1990). Baiting techniques combined with molecular diagnostic methods have been successfully utilized in detecting several *Phytophthora* species from soil (Nechwatal et al. 2001; Sutton et al. 2007). When DNA from infected baiting tissues are extracted and used for PCR, the pathogen present in a given sample is concentrated as it multiplies and produces more mycelium and possibly other infectious units compared to an extraction from soil. Hence, combining both methods should increase the sensitivity of pathogen detection vs. using bait testing alone. Co-infection by other organisms (e.g. *Pythium* sp.) will not affect the procedure when specific primer pairs not showing any cross-reactions with various soil fungi are used (Nechwatal et al. 2001). Several conventional and real-time PCR protocols with different levels of specificity and sensitivity have been developed to detect *P. erythroseptica* in tuber tissue. These protocols are based on the primers designed from internal transcribed spacer (ITS) regions of ribosomal DNA (Tooley et al. 1997; Cullen et al. 2007).

Several plant tissue types reported as baits for *Phytophthora* spp. were evaluated as potential baits for *P. erythroseptica* in preliminary experiments. These plant tissue types were floated on zoospore suspensions and symptom development was observed overtime. Among those evaluated were shore juniper (*Juniperus conferta*) and eastern white pine needles (*Pinus strobus* L.), mustard (*Brassica juncea* (L.) Czern.) and lupine (*Lupinus* sp.) cotyledons, radish (*Raphanus sativus* L.), tomato (*Solanum lycopersicum* L.) and fenugreek (*Trigonella foenum-graecum* L.) seedlings, leaf discs of potatoes (Shepody), eucalyptus (*Eucalyptus* sp.) leaves, rye (*Secale cereale* M.Bieb.) seeds, tomato stems from mature plants, hairy nightshade (*Solanum sarrachoides* Sendt.) and bitter nightshade (*Solanum dulcamara* L.) cotyledon leaves. Of the different plant tissue types screened, clear symptoms developed only on hairy nightshade and bitter nightshade cotyledon leaves. Potato leaf discs deteriorated quickly and therefore excluded from further study.

Hairy nightshade is a common weed species found in potato production fields while bitter nightshade is generally found in the North American potato growing areas. Porter et al. (2007) reported hairy nightshade to be a notable host for *P. erythroseptica* based on artificial inoculations in the greenhouse. The objective of this study was to evaluate hairy and bitter nightshade for their ability to bait *P. erythroseptica* from infested soil samples using traditional baiting techniques and improve detection using species-specific PCR primers.

Materials and Methods

Plant Materials and Pathogen Cultures

Hairy Nightshade seeds collected in 2006 were obtained from Dr. Pamela Hutchinson (Aberdeen research and extension center, University of Idaho, USA). Ripe bitter nightshade berries were collected from Cavendish farm, Prince Edward Island, Canada and seeds extracted by

grinding in a blender and filtering using cheesecloth. Hairy and bitter nightshade seeds were sown about 1 cm deep in flats (53 cm×27.5 cm×5.5 cm) filled with potting mixture (ASB-Green world Ltd., Pointe Sapin, New Brunswick, Canada) and placed in the greenhouse maintained at 25–30ºC. The flats were kept moist at all times. Seeds germinated in 6–10 days and 13–15 days for hairy nightshade and bitter nightshade, respectively. Zoospore suspensions were prepared by stimulating *P. erythroseptica* (isolate PE9913-2DSZ1, from Prince Edward Island, Canada) to produce sporangia/zoospores using the protocol described by Peters et al. (2001). Zoospore suspensions were standardized using a hemacytometer to 50,000 and 20,000 zoospores/ml for subsequent trials. *Phytophthora erythroseptica* DNA for the positive control was obtained from pure cultures using the protocol described by Peters et al. (2005). *Phytophthora infestans* DNA for the negative control was obtained from pure cultures using the method described by Goodwin et al. (1992).

Use of Hairy and Bitter Nightshade for Detection of *Phytophthora erythroseptica*

Evaluation of Hairy and Bitter Nightshade as Baits for Phytophthora erythroseptica

Phytatrays (Sigma-Aldrich Co.) which are disposable cell culture vessels (11.3 cm×10 cm×10.5 cm) were filled with 200 ml of zoospore suspensions (39×10^4 zoospores/ml) or sterile distilled water as the non-inoculated control. Hairy nightshade and bitter nightshade cotyledon leaves and two leaf stage (TLS) seedlings (seedlings with two true leaves and two cotyledon leaves) were evaluated in separate trials. All seedlings were rinsed with tap water to remove debris s attached to the roots before suspending on Styrofoam beads (used in shipping by commercial couriers (approximately 3.5 cm×2 cm) as shown in Fig. 1a. The cotyledon leaves were also rinsed with tap water and floated on zoospore suspensions. The phytatrays were incubated in a growth chamber at 18ºC under fluorescent lamps for a 16 h photoperiod. Each tissue type was incubated in two phytatrays with six baits in each phytatray and the trial was repeated once. DNA was extracted from the bait tissues 7 days after incubation in *P. erythroseptica* zoospore suspension, and presence of pathogen was determined.

Determination of the Length of Incubation Period for Hairy and Bitter Nightshade Tissue Types

Phytatrays were filled with 200 ml of the standardized zoospore suspensions (20,000 and 50,000 zoospores/ml) or sterile distilled water as the non-inoculated control. Hairy nightshade and bitter nightshade TLS seedlings, mature seedlings with four true leaves or more (FLS), true leaves and cotyledon leaves were evaluated in separate trials. The seedling roots were rinsed with tap water and suspended on Styrofoam beads while the leaves were floated in zoospore suspensions. Each tissue type was evaluated in two separate phytatrays with five samples in each phytatray. The trials were repeated once. The phytatrays were incubated in a growth chamber as described above. One sample was removed from each phytatray after 2, 4, 6, 8 and 10 days of incubation. DNA was extracted from the bait tissue types and the presence of *P. erythroseptica* determined.

Detection of Phytophthora erythroseptica from Artificially Inoculated Soils using Hairy and Bitter Nightshade Tissues

Field soil collected from potato fields in New Brunswick was sterilized in sealed bio-hazard bags by autoclaving at 120ºC for 20 min. After 2 days, the bags were re-autoclaved under the same conditions and allowed to cool overnight. Phytatrays were filled with 100 g of sterilized field soils and mixed with 200 ml of standardized zoospore suspensions (20,000 and 50,000 zoospores/ml). The sterile distilled water mixed with 100 g of sterilized field soil served as the non-inoculated control. TLS seedlings, FLS seedlings, true leaves and cotyledon leaves of hairy and bitter nightshade tissue were evaluated. The seedling roots were rinsed with tap water and then suspended on styrofoam beads while making sure that the roots did not come in direct contact with the soils. The leaves were also washed with tap water and floated. The phytatrays were incubated as described above. Each bait tissue was evaluated in two separate phytatrays with five samples in each phytatray. The trials were repeated once. One sample was removed from each phtyatray after 2, 4, 6, 8 and 10 days of incubation. DNA was extracted from the bait tissue and the presence of *P. erythroseptica* determined.

Detection of Phytophthora erythroseptica from Flooded Naturally Infested Field Soils using Hairy and Bitter Nightshade as Tissues

Hairy and bitter nightshade cotyledon leaves and TLS seedlings were evaluated using field soil known to be infested with *P. erythroseptica* (Wicklow, New Brunswick, Canada). Sterilized field soil (100 g) was mixed with 200 ml of sterile distilled water in phytatrays as the non-inoculated control. The phytatrays were incubated in a growth chamber as described above. Each bait tissue was evaluated in two separate phytatrays with five samples in each phytatray. The trials were repeated once. One sample was removed from each phtyatray after 2, 4, 6, 8 and 10 days of incubation. DNA was extracted from

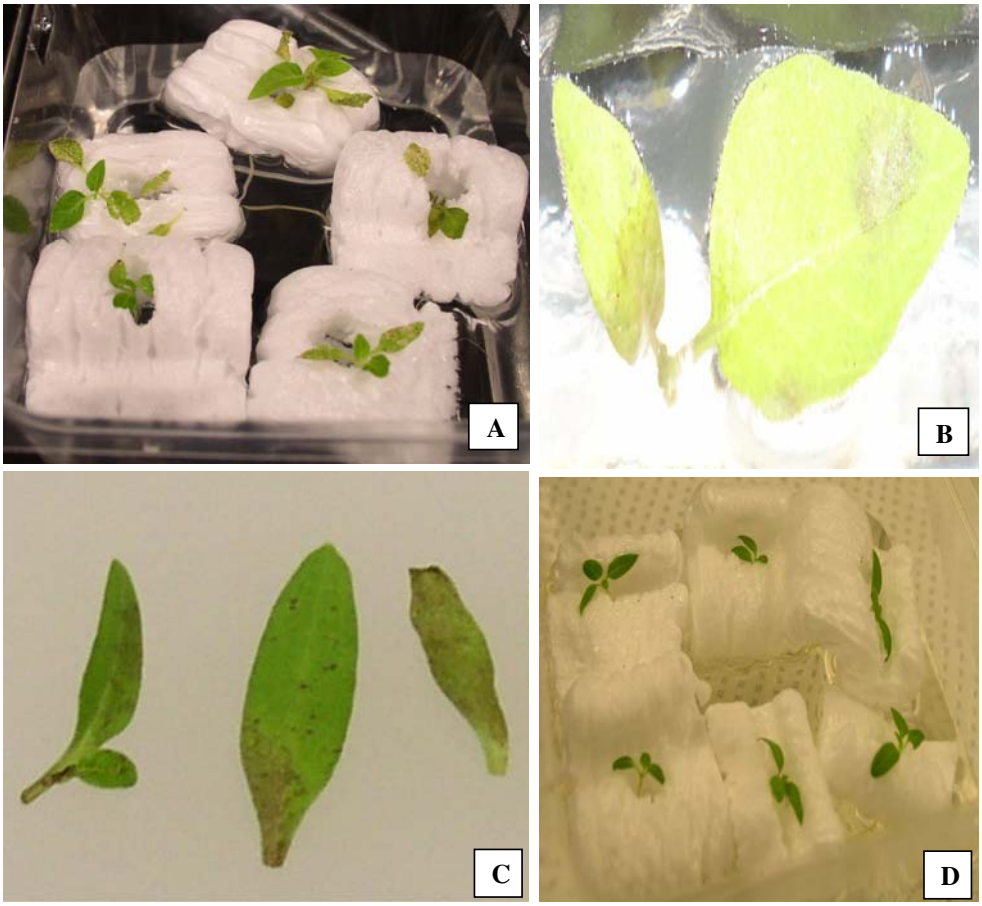

Fig 1 Symptoms on hairy nightshade two leaf stage seedlings suspended on styrofoam beads **a** and cotyledon leaves **b** and bitter nightshade cotyledon leaves **c** incubated in zoospore suspension (39×10^4 zoospores/ml) and hairy nightshade two leaf stage seedlings incubated in the non-inoculated control **d** for 7 days

the bait tissue types and the presence of *P. erythroseptica* determined. DNA was also extracted directly from six infested field soil samples using a Mobio-ultra clean soil DNA kit (Mobio laboratories Inc., Carlsbad, CA, USA) following manufacturer's instructions.

PCR Protocols

Entire seedlings and the leaves were ground using a pre-chilled mortar and pestle with a pinch of acid washed sea sand (Fischer Scientific, Fairlawn, New Jersey, USA). DNA was extracted from 100 mg of ground tissue using a Qiagen DNeasy plant mini-kit (Qiagen Sciences, Maryland, USA) following the manufacturer's instructions. Only 50 µl of elution buffer was used in the final step in order to increase DNA concentration. The presence of *P. erythroseptica* was determined using conventional and real-time PCR methods as described below.

Conventional PCR

The PCR method developed by Tooley et al. (1997) was modified to increase the sensitivity of the assay as described by Nanayakkara et al. (2009). PCR amplification of samples was based on an initial denaturation at 94ºC for 30 s, followed by 36 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, extension at 72ºC for 1 min, and a final elongation at 72ºC for 4 min in a reaction volume of 25 µl using a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA). Optimal conditions for PCR contained a master mix of the following components: 1×PCR buffer, 4 mM $MgCl_2$, 0.5 units of *Taq* Polymerase (AmpliTaq DNA polymerase with GeneAmp, Applied Biosystems, Foster City, CA, USA), 140 µM each dNTPs (Promega, Madison, USA) and 0.2 µM PERY2 and ITS4 primers (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences of the primers PERY2 and ITS4 are given in Table 1. Undiluted DNA (2 µl) was used as the template in 23 µl of master mix. DNA extracted from pure cultures of *P. erythroseptica* (isolate PE9913-2DSZ1 from Prince Edward Island, Canada) was used as the positive control while DNAse free water and non-target DNA from *Phytophthora infestans* (Mont.) de Bary (isolate 2006-072 from New Brunswick, Canada) served as the negative control. PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide and photographed under UV illumination (Fisher Biotech Electrophoresis System, Fisher Scientific, Pittsburgh, USA). Low DNA mass ladder was used as the molecular

Table 1 Nucleotide sequences of the PCR primers and probes used in this study

Primer	Sequence	Source
PERY2	CTGTTCCGGCGTAAGCTGG	Tooley et al. 1997
ITS4	TCCTCCGCTTATTGATATGC	Tooley et al. 1997
99F	TGTGCTAGGCTTGGCGTTT	Cullen et al. 2007
177R	CCTCGTCCACCCCAGCTTA	Cullen et al. 2007
133T[a]	TGCGAAGTAGGGTGTGTTCCGGC	Cullen et al. 2007

[a] Probe 133 T was labeled at the 5'-end with FAM (6-carboxy-fluorescein) and 3'-end with the quencher dye TAMRA

weight standard. The PCR assay yielded a 136 bp product in the presence of the target template.

Real-time PCR

The real-time PCR method developed by Cullen et al. (2007) and as described by Nanayakkara et al. (2009) was performed in MicroAmp optical 96-well plates using ABI 7000 sequence detectors (Applied Biosystems, Foster City, USA) following the recommended generic (three stage) thermal cycle protocol. Real-time PCR reaction involved the addition of 1 µl of target template to an optimized mix consisting of TaqMan Buffer (1×), 3.5 mM $MgCl_2$, 0.5 units Ampli*Taq* Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 0.2 mM each of dNTPs (Promega, Madison, USA), 100 nM probe 133T and 300 nM each of forward 99F and reverse primers 177R (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences of the probe 133T and the primers 99F and 177R are given in Table 1. The PCR assay generated a 78 bp product in the presence of the target template.

Results

Hairy nightshade TLS seedlings developed chlorosis and brown lesions on the roots and wilted 7 days after incubation in zoospore suspensions (39×10^4 zoospores/ml) (Fig. 1a). The cotyledon leaves also developed browning on the stem end and water soaked spots (Fig. 1b). 40% of bitter nightshade TLS seedlings also developed foliar chlorosis while 80% of the cotyledon leaves had brown specks 7 days after incubation (Fig. 1c). TLS seedlings and cotyledon leaves of hairy and bitter nightshades incubated in the non-inoculated control did not develop any symptoms (Fig 1d), and pathogen was not detected in any of control plants. *Phytophthora erythroseptica* was detected in all replicates of TLS seedlings and cotyledon leaves of pathogen treated hairy and bitter nightshades by using both conventional and real-time PCR methods (Fig. 2) and in both trials.

Phytophthora erythroseptica was detected in hairy nightshade cotyledon leaves and TLS seedlings incubated in both 20,000 and 50,000 zoospores/ml suspension and amended flooded soil (Tables 2 and 3). Earliest detection was in cotyledon leaves incubated for 2 days in 50,000 zoospores/ml suspension (Tables 2 and 4), whereas 10 days after incubation in soils amended with both concentrations (Tables 3 and 5). *P. erythroseptica* was detected in TLS seedlings 4 days after incubation in 50,000 zoospores/ml suspension with the real-time PCR assay (Table 2) but 6 days in soils amended with 20,000 zoospores/ml with both PCR assays (Table 3). The conventional PCR assay did not detect the pathogen in hairy nightshade TLS seedlings or cotyledon leaves before 6 days of incubation (Table 2). Symptoms were first observed in TLS seedlings and cotyledon leaves after incubating for 6 days at both concentrations. The pathogen was also detected with both conventional and real-time PCR assays in cotyledon leaves and TLS seedlings 6 and 10 days after incubation in flooded naturally infested field soil, respectively. *Phytophthora erythroseptica* was not detected in hairy nightshade true leaves or FLS seedlings incubated in either suspension or flooded soil at both concentrations tested. *Phytophthora erythroseptica* was not detected in hairy nightshade tissue types incubated in the non-inoculated control with either assay.

Phytophthora erythroseptica was detected in bitter nightshade cotyledon leaves incubated in both 20,000 and 50,000

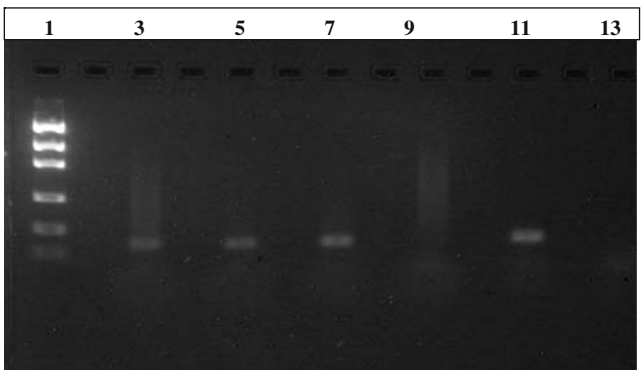

Fig. 2 Detection of *P. erythroseptica* in hairy nightshade two leaf stage seedlings incubated in zoospore suspension and sterile distilled water with the conventional PCR. Lane 1. DNA mass ladder; Lanes 3–7. Hairy nightshade two leaf stage seedlings (TLS) incubated in zoospore suspension (39×10^4 zoospores/ml); Lane 9. Hairy nightshade TLS seedlings incubated in Sterile distilled water (SDW); Lane 11. Positive control (DNA from pure cultures of *P. erythroseptica*); 13. Negative control. The non-numbered even lanes were left empty

Table 2 Detection of *Phytophthora erythroseptica* in hairy and bitter nightshade tissue types incubated in zoospore suspensions using PCR and real-time PCR assays

Common name	Zoospore concentration spores/ml	Bait tissue	Number of samples positively detected with PCR/real-time PCR				
			Incubation period				
			2 days n=2	4 days n=2	6 days n=2	8 days n=2	10 days n=2
Hairy nightshade	20,000	TLS seedlings					
		Exp 1	0/0	0/0	1/1	1/1	1/2
		Exp 2	0/0	0/0	0/0	1/1	2/2
		Cotyledon leaves					
		Exp 1	0/0	0/0	1/1	0/1	2/2
		Exp 2	0/0	0/0	0/1	1/1	2/2
		FLS seedlings					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
		True leaves					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
	50,000	TLS seedlings					
		Exp 1	0/0	0/1	0/0	0/2	1/2
		Exp 2	0/0	0/1	0/0	1/2	0/2
		Cotyledon leaves					
		Exp 1	0/1	0/0	1/2	0/2	2/2
		Exp 2	0/0	0/0	0/1	0/2	1/2
		FLS seedlings					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
		True leaves					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
Bitter nightshade	20,000	TLS seedlings					
		Exp 1	0/0	0/0	0/0	1/2	0/2
		Exp 2	0/0	0/0	0/0	0/1	0/2
		Cotyledon leaves					
		Exp 1	0/0	0/0	0/0	0/0	0/2
		Exp 2	0/0	0/0	0/0	0/0	0/2
		FLS seedlings					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
		True leaves					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
	50,000	TLS seedlings					
		Exp 1	0/0	0/0	0/0	0/0	0/1
		Exp 2	0/0	0/0	0/0	0/0	0/2
		Cotyledon leaves					
		Exp 1	0/0	0/1	0/0	1/1	1/1
		Exp 2	0/0	0/0	0/1	1/1	1/2
		FLS seedlings					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0

Table 2 (continued)

Common name	Zoospore concentration spores/ml	Bait tissue	Number of samples positively detected with PCR/real-time PCR				
			Incubation period				
			2 days n=2	4 days n=2	6 days n=2	8 days n=2	10 days n=2
		True leaves					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0

The bait tissue incubated in the non-inoculated control tested negative at all sampling times

Table 3 Detection of *Phytophthora erythroseptica* in hairy and bitter nightshade tissue types incubated in flooded-infested soils using PCR and real-time PCR assays

Common name	Soil inoculum zoospores/ml	Bait tissue	Number of samples positively detected with PCR/real-time PCR				
			Incubation period				
			2 days n=2	4 days n=2	6 days n=2	8 days n=2	10 days n=2
		TLS seedlings					
Hairy nightshade	20,000	Exp 1	0/0	0/0	1/1	1/2	1/1
		Exp 2	0/0	0/0	1/1	1/2	1/2
		Cotyledon leaves					
		Exp 1	0/0	0/0	0/0	0/0	1/1
		Exp 2	0/0	0/0	0/0	0/0	0/1
		FLS seedlings					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
		True leaves					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
		TLS seedlings					
	50,000	Exp 1	0/0	0/0	0/0	0/1	0/2
		Exp 2	0/0	0/0	0/0	0/0	1/2
		Cotyledon leaves					
		Exp 1	0/0	0/0	0/0	0/0	0/1
		Exp 2	0/0	0/0	0/0	0/0	0/2
		FLS seedlings					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
		True leaves					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
		TLS seedlings					
	Naturally infested soils	Exp 1	0/0	0/0	0/0	0/0	1/1
		Exp 2	0/0	0/0	0/0	0/0	1/2
		Cotyledon leaves					
		Exp 1	0/0	0/0	0/1	1/1	1/2
		Exp 2	0/0	0/0	1/1	1/2	1/2

Table 3 (continued)

Common name	Soil inoculum zoospores/ml	Bait tissue	Number of samples positively detected with PCR/real-time PCR				
			Incubation period				
			2 days n=2	4 days n=2	6 days n=2	8 days n=2	10 days n=2
Bitter nightshade	20,000	TLS seedlings					
		Exp 1	0/0	0/0	0/1	0/1	1/1
		Exp 2	0/0	0/0	1/1	0/1	1/2
		Cotyledon leaves					
		Exp 1	0/0	0/1	0/0	1/1	1/2
		Exp 2	0/0	0/0	0/0	1/1	1/1
		FLS seedlings					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
		True leaves					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
		TLS seedlings					
	50,000	Exp 1	0/0	0/0	0/0	0/0	0/1
		Exp 2	0/0	0/0	0/0	0/0	0/2
		Cotyledon leaves					
		Exp 1	0/0	0/0	0/0	0/1	0/2
		Exp 2	0/0	0/0	0/0	0/1	0/1
		FLS seedlings					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
		True leaves					
		Exp 1	0/0	0/0	0/0	0/0	0/0
		Exp 2	0/0	0/0	0/0	0/0	0/0
		TLS seedlings					
	Naturally infested soils	Exp 1	0/0	0/0	0/0	0/0	1/1
		Exp 2	0/0	0/0	0/0	0/0	1/2
		Cotyledon leaves					
		Exp 1	0/1	0/1	0/1	1/1	1/2
		Exp 2	0/0	0/1	1/1	1/2	0/2

The bait tissue incubated in the non-inoculated control tested negative at all sampling times

zoospores/ml (Table 2) with both PCR assays. However, the pathogen was detected from the TLS seedlings incubated in 50,000 zoospores/ml only with the real-time PCR assay (Table 4). In amended flooded soil *P. erythroseptica* was detected in TLS seedlings and in cotyledon leaves at both concentrations (Table 3). Earliest detection was in cotyledon leaves incubated for 4 days in 50,000 zoospores/ml suspension only with the real-time PCR as compared to TLS seedlings 8 days after incubation in 20,000 zoospores/ml suspension with both PCR assays (Table 2). However, earliest detection in cotyledon leaves and TLS seedlings incubated in soils amended with 20,000 zoospores/ml were 4 and 6 days, respectively (Table 3 and 5). *Phytophthora erythroseptica* was also detected in flooded naturally infested field soil in bitter nightshade cotyledon leaves 2 days after incubation only with the real-time PCR assay as compared to 10 days after incubation in TLS seedlings with both PCR assays. The pathogen was not detected in true leaves or FLS seedlings incubated in zoospore suspensions or flooded infested soils. *Phytophthora erythroseptica* was not detected in any bitter nightshade bait tissue incubated in the non-inoculated control with either assay.

Table 4 Ct values of detection for *Phytophthora erythroseptica* in hairy and climbing nightshade leaves and seedling bait tissues incubated in zoospore suspensions using real-time PCR assay

Common name	Zoospore concentration spores/ml	Bait tissue	CT values*				
			Incubation period				
			2 days	4 days	6 days	8 days	10 days
Hairy nightshade	20,000	TLS seedlings	–	–	19.0	24.0	23.2
		Cotyledon leaves	–	–	19.0	23.3	28.1
	50,000	TLS seedlings	–	20.0	–	23.5	25.3
		Cotyledon leaves	28.3	–	19.5	20.6	25.0
Bitter nightshade	20,000	TLS seedlings	–	–	–	29.0	30.4
		Cotyledon leaves	–	–	–	–	32.0
	50,000	TLS seedlings	–	–	–	–	33.0
		Cotyledon leaves	–	30.0	33.5	34.0	32.5

The bait tissue incubated in the non-inoculated control tested negative at all sampling times; *CT Values are average of number of positives detected in Table 2

– undetected

The pathogen was not detected in the DNA extracted from any of the infested field soil samples with either PCR assay.

Discussion

Hairy and bitter nightshade TLS seedlings and cotyledon leaves successfully baited *P. erythroseptica* from flooded infested soils. The pathogen was detected with both PCR assays. These baits also detected pathogen from all three concentrations of zoospores. However, it is difficult to extrapolate this information to infested potato fields as propagules of *P. erythroseptica* in field soil have never been quantified. Further work is required to establish the minimum number of *P. erythroseptica* propagules in field soils that this bioassay procedure can detect. Baiting indicates that not only are the pathogen propagules present but they are also viable in the soil. The pathogen was not

Table 5 Ct values of detection for *Phytophthora erythroseptica* in hairy and climbing nightshade leaves and seedling bait tissues incubated in flooded-infested soils using real-time PCR assay

Common name	Soil Inoculum zoospores/ml	Bait tissue	CT values*				
			Incubation period				
			2 days	4 days	6 days	8 days	10 days
Hairy nightshade	20,000	TLS seedlings	–	–	23.6	30.5	24.6
		Cotyledon leaves	–	–	–	–	34.0
	50,000	TLS seedlings	–	–	–	33.5	33.3
		Cotyledon leaves	–	–	–	–	33.5
	Naturally infested soils	TLS seedlings	–	–	–	–	34.0
		Cotyledon leaves	–	–	31.7	22.7	31.9
Bitter nightshade	20,000	TLS seedlings	–	–	32.5	30.6	32.9
		Cotyledon leaves	–	28.5	–	28.4	31.9
	50,000	TLS seedlings	–	–	–	–	29.1
		Cotyledon leaves	–	–	–	31.8	30.8
	Naturally infested soils	TLS seedlings	–	–	–	–	32.7
		Cotyledon leaves	24.0	29.7	34.6	32.0	29.5

The bait tissue incubated in the non-inoculated control tested negative at all sampling times; *CT Values are average of number of positives detected in Table 3

– undetected

detected by the DNA extracted from the same infested soils. This may be because the pathogen propagules were not uniformly distributed in the soil samples. Therefore, baiting with susceptible tissue types may enhance detection of *P. erythroseptica* in soil. Dance et al. (1975) reported that greater quantities of soil can be assayed using baiting methods, thereby increasing the likelihood of detecting populations present at low densities. Flooding infested soils mimics the disease cycle where zoospores infect the tubers or roots of potato plants. The real-time PCR assay was clearly more sensitive than the conventional PCR assay. Cullen et al. (2007) reported that the real-time PCR assay reliably detected atto gram (10^{-18} g) levels of target DNA from pure cultures. This bio-assay can be easily performed in a laboratory setting and could be a valuable tool for research and routine diagnosis. Hairy and bitter nightshade bait plants can be easily grown in a greenhouse from seeds collected during the previous growing season.

Davoren and Wicks (1999) reported that camellia (*Camellia japonica*) leaf discs and tomato seedlings could successfully bait *P. erythroseptica* from naturally infested soils from potato fields. Detection levels were highest after 24–48 h of baiting. However, 3–5 days of growth on a nutrient medium was necessary to identify the pathogen. The sensitivity of the baits was not determined in this study. By combining baiting with PCR, we avoided the difficulties of direct isolation from baits followed by identification of species. PCR also increased the precision of the bait test. However, time was still required for *P. erythroseptica* zoospores to infect and develop on the bait tissues. Generally 24–48 h is accepted as the optimum duration for baiting many species of *Phytophthora* (Ferguson and Jeffers 1997) including *P. erythroseptica* (Davoren and Wicks 1999). Although detection was made in some cotyledon leaves and TLS seedlings earlier than 10 days, incubation for a minimum of 10 days is recommended for reliable results. This bioassay procedure successfully detected *P. erythroseptica* propagules in field soil. The ability to detect viable pathogen propagules in soil could be a valuable management tool that enables growers to plan control strategies accordingly. Further investigations are recommended to compare camellia leaf discs and tomato roots with hairy nightshade and bitter nightshade bait tissue using the bio-assay procedure developed in this study.

Phytophthora erythroseptica has not been observed to infect hairy and bitter nightshade plants in the field. In this study, hairy nightshade and bitter nightshade TLS seedlings became infected and the pathogen was detected when suspended in flooded infested soils. However, *P. erythroseptica* was not detected in any hairy nightshade or bitter nightshade FLS seedlings suspended in flooded infested soils indicating a change in susceptibility with tissue maturity. Further investigations are recommended to evaluate the role of hairy nightshade and bitter nightshade plants as weed hosts for *P. erythroseptica*.

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