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"Comparison of methods to detect resistance of *Helminthosporium solani* to a thiabendazole fungicide"

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Phytoprotection, vol. 82, n° 3, 2001, p. 103-112.

Comparison of methods to detect resistance of *Helminthosporium solani* to a thiabendazole fungicide[1]

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Received 2001-09-11; accepted 2002-01-22

PHYTOPROTECTION 82 : 103-112

The development of resistance to a post-harvest and preplanting benzimidazole fungicide, thiabendazole (TBZ), has resulted in the increase of silver scurf of potato caused by *Helminthosporium solani*. Conventional and polymerase chain reaction (PCR)-based assays were compared, among 54 isolates of *H. solani*, to detect the TBZ-sensitive (TBZ^S) and -resistant (TBZ^R) isolates. In a PCR-based assay, all 27 single-spore isolates from eastern Canada, nine isolates from Alberta in western Canada, six isolates from North Dakota, USA were distinguished as TBZ^R, the remainder of the isolates being determined as TBZ^S. The results from the PCR-based assay correlated well with the TBZ sensitivity assessment of isolates of *H. solani* on the TBZ-amended medium. Also, the detection of TBZ^R-*H. solani* in 20 tuber samples with silver scurf lesions by the PCR-based assay demonstrates that this method can be used to detect the pathogen directly from the tuber lesions. The PCR-based method was more rapid (1 day) than the conventional technique (5 weeks).

[Comparaison de méthodes pour détecter la résistance de *Helminthosporium solani* au fongicide thiabendazole]

Le développement de la résistance à un fongicide de benzimidazole, le thiabendazole (TBZ), utilisé après la récolte et avant la plantation, a eu comme conséquence l'augmentation de l'incidence de la tache argentée de la pomme de terre causée par *Helminthosporium solani*. Des analyses conventionnelles ou basées sur la réaction de polymérisation en chaîne (PCR) ont été comparées pour détecter, parmi 54 isolats de *H. solani*, les isolats sensibles (TBZ^S) et résistants (TBZ^R) au TBZ. L'analyse basée sur la PCR a déterminé les 27 isolats simples de spores de l'est du Canada, neuf des isolats de l'Alberta (ouest du Canada) et six des isolats du North Dakota (États-Unis) comme TBZ^R, le reste des isolats étant TBZ^S. Les résultats de

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l'analyse basée sur la PCR ont été bien corrélés avec l'évaluation de la sensibilité au TBZ des isolats de *H. solani* sur le support avec amendement au TBZ. En outre, la détection de TBZ^R-*H. solani* dans 20 échantillons de tubercules infectés par la tache argentée par l'analyse basée sur la PCR démontre que cette méthode peut être utilisée dans la détection de l'agent pathogène directement à partir des lésions infectées du tubercule. La méthode basée sur la PCR était plus rapide (1 jour) que la technique conventionnelle (5 semaines).

INTRODUCTION

Silver scurf of potato (*Solanum tuberosum* L.), caused by the fungal pathogen *Helminthosporium solani* Durieu & Montagne, has increased in economic importance from a number of factors that include resistance of the fungus to thiabendazole fungicides, storage of tubers at higher humidities, lowered defect tolerances, and increased awareness by the industry (Errampalli *et al.* 2001a; Secor and Gudmestad 1999). The disease affects the quality of all market categories of potatoes. The silver to tan to gray lesions on the tubers, detract from the appearance of tubers, cause excessive fresh weight losses due to an increased water permeability of the tuber skin, and thickened skin results in undesirable burnt edges around the chips after cooking. These lesions lead to the rejection of tubers, and thus cause economic losses (Boyd 1972; Errampalli *et al.* 2001a; Holley and Kawchuck 1996; Jellis and Taylor 1977; Rodriguez *et al.* 1996).

Silver scurf was effectively controlled by thiabendazole (TBZ), a benzimidazole fungicide, until 1977 when TBZ-resistant (TBZ^R) isolates of *H. solani* were identified in stored tubers, in which TBZ had been used as a post-harvest treatment (Hide *et al.* 1988). The presence of TBZ^R isolates of *H. solani* was reported in many commercial seed storages in Canada (Bains *et al.* 1996; Errampalli 2000; Errampalli *et al.* 2001a; Holley and Kawchuck 1996; Kawchuck *et al.* 1994; Platt 1997; Szeto *et al.* 1993), the UK (Hide *et al.* 1988), Sweden (Bang 1993), and the US (Rodriguez *et al.* 1996). TBZ^R isolates of *H. solani* were found in soils in commercial storages where TBZ was used as a post-harvest treatment (Frazier *et al.* 1998; Errampalli, unpublished). The isolates of *H. solani* present in areas which had never been treated with TBZ before, were all TBZ^S (Hide *et al.* 1988).

The mode of action of benzimidazoles, including TBZ, is that the chemical compound binds to fungal β-tubulin protein and causes the inhibition of fungal microtubule function (Davidse and Flach 1978). The binding affinity of TBZ to β-tubulin *in vitro* is directly related to the sensitivity of the isolates to these fungicides (Koenraadt *et al.* 1992). Resistance to TBZ in isolates of *H. solani* results from a point mutation in the β-tubulin gene in codon 198 or 200 that causes an altered amino acid sequence at the TBZ binding site (McKay and Cooke 1997).

Rapid detection allows the monitoring of TBZ^R isolates of *H. solani* and enables crop managers to implement suitable strategies to manage silver scurf in a timely fashion. Some of the alternative management strategies to the use of TBZ were reviewed (Errampalli *et al.* 2001a). The use of seed tubers free of silver scurf is not feasible because most of the seed tubers are infected with *H. solani* (Secor and Gudmestad 1999). A periodic removal of TBZ fungicide and alternation with a fungicide other than the bezimidazoles in the disease control program may help to eliminate the TBZ^R populations and may help to retain sensitivity to TBZ in the remaining populations of *H. solani*.

The available method to detect TBZ^R isolates of *H. solani*, is to culture the isolates on fungicide-amended nutrient media and to measure the growth response to TBZ (Hide *et al.* 1988; Holley and Kawchuck 1996; Platt 1997). This method is generally tedious and time consuming as it takes 4-5 wk. Poly-

merase chain reaction (PCR)-based methods developed to detect and identify plant pathogenic fungi are fast and sensitive (Edel 1998). A PCR assay, developed on the basis of a mutation in β-tubulin of TBZ^R isolates of *H. solani*, was successfully used to detect TBZ^R isolates of *H. solani* in tuber samples collected from Northern Ireland (McKay and Cooke 1997).

The objective of this study was to compare the conventional method with that of the PCR assay designed by McKay and Cooke (1997), and to identify a rapid detection method for TBZ^R isolates of *H. solani* collected from Canada. Preliminary results have been published (Errampalli 2000).

MATERIALS AND METHODS

Fungal isolates, tubers with silver scurf and soils

Isolates of *H. solani* used in this study and their geographical origins are listed (Table 1). All isolates were routinely grown on potato dextrose agar (PDA, Difco laboratories, Detroit, MI) at 22°C. For long-term storage, isolates were grown on half-strength PDA and stored at 4°C. Prior to each test, isolates were subcultured onto fresh PDA from colonized agar plugs (5-mm diam).

Single-spore isolates of *H. solani* were obtained from potato tubers with silver scurf from a potato-store cell at the Agriculture and Agri-Food Canada's

Table 1. Origin of isolates of *Helminthosporium solani* used in this study and the determination of thiabendazole (TBZ)-resistant and -sensitive *H. solani* by conventional detection method and PCR-based assay

No.	Isolate of H. solani[a]	Origin		Conventional method[c]	PCR-based assay[d]
		Year	Location[b]		
1	PHsD1b	1999	PEI, Canada[e]	R[f]	R
2	PHsD2b	1999	PEI, Canada	R	R
3	PHsD3b	1999	PEI, Canada	R	R
4	PHsD4b	1999	PEI, Canada	R	R
5	PHsD6b	1999	PEI, Canada	R	R
6	PHsD7b	1999	PEI, Canada	R	R
7	PHsD8b	1999	PEI, Canada	R	R
8	PHsD9b	1999	PEI, Canada	R	R
9	PHsD10b	1999	PEI, Canada	R	R
10	PHsD11a	1999	PEI, Canada	R	R
11	PHsD12b	1999	PEI, Canada	R	R
12	PHsD13b	1999	PEI, Canada	R	R
13	PHsD14b	1999	PEI, Canada	R	R
14	PHsD15b	1999	PEI, Canada	R	R
15	PHsD16b	1999	PEI, Canada	R	R
16	PHsD17b	1999	PEI, Canada	R	R
17	PHsD18b	1999	PEI, Canada	R	R
18	PHsD19a	1999	PEI, Canada	R	R
19	PHsD20b	1999	PEI, Canada	R	R
20	PHsD21a	1999	PEI, Canada	R	R
21	PHsD22b	1999	PEI, Canada	R	R
22	PHsD23a	1999	PEI, Canada	R	R
23	PHsD24b	1999	PEI, Canada	R	R
24	PHsD25a	1999	PEI, Canada	R	R
25	PHsD26a	1999	PEI, Canada	R	R
26	PHsD27a	1999	PEI, Canada	R	R
27	PHsD28b	1999	PEI, Canada	R	R
28	AB25-B1	N/A[g]	AB, Canada[h]	S[i]	S
29	AB29-B1	N/A	AB, Canada	R	R
30	AB65-A1	N/A	AB, Canada	R	R

Table 1. Origin of isolates of *Helminthosporium solani* used in this study and the determination of thiabendazole (TBZ)-resistant and -sensitive *H. solani* by conventional detection method and PCR-based assay (cont'd)

No.	Isolate of H. solani[a]	Origin		Conventional method[c]	PCR-based assay[d]
		Year	Location[b]		
31	AB107-B2	N/A	AB, Canada	R	R
32	AB185-A2	N/A	AB, Canada	R	R
33	AB338-A1	N/A	AB, Canada	S	S
34	AB455-A1	N/A	AB, Canada	S	S
35	AB455-B1	N/A	AB, Canada	S	S
36	AB468-A2	N/A	AB, Canada	R	R
37	AB479-A1	N/A	AB, Canada	S	S
38	AB517-D3	N/A	AB, Canada	S	S
39	AB527-A1	N/A	AB, Canada	R	R
40	AB632-C1	N/A	AB, Canada	R	R
41	NC 2B	N/A	AB, Canada	R	R
42	NC 9A	N/A	AB, Canada	R	R
43	ND1-21	1997	ND, USA[j]	R	R
44	ND3-21(R)	1995	ND, USA	R	R
45	ND3-24(R)	1995	ND, USA	R	R
46	ND23-2	1996	ND, USA	S	S
47	ND23-10	1996	ND, USA	R	R
48	ND23-14	1996	ND, USA	S	S
49	ND23-24	1996	ND, USA	R	R
50	ND24-13	1996	ND, USA	R	S
51	ND24-21	1996	ND, USA	R	S
52	ND24-24	1996	ND, USA	R	R
53	ND24-25	1996	ND, USA	S	S
54	ND28-12	1998	ND, USA	S	S

[a] All isolates were collected from potato (*Solanum tuberosum* L.) and were confirmed as *H. solani* by both PCR amplification of 447 bp fragment with primer pairs, Hs1F1 and Hs2R1, and colony morphology, conidiophore and conidial structure by the method of Errampalli *et al.* (2001b).

[b] location: PEI = Prince Edward Island, Canada; AB = Alberta, Canada; ND = North Dakota, USA.

[c] Growth of each of the isolates on culture medium with 0, 5, 10, 20, and 50 μg ml^{-1} TBZ was recorded weekly for 5 weeks. Also three isolates AB29-B1, AB107-B2, and AB185-A2 were grown on 0,5, 100, 250, and 500 μg ml^{-1} TBZ. Based on growth rates, the isolates were determined as either R (TBZ-resistant) or S (TBZ-sensitive).

[d] All isolates were tested with PCR amplification of 872 bp fragment with primer pairs, SS-for and SS-rev, and digestion with a restriction endonuclease, *Bsa*I.

[e] Isolates from PEI were collected by D. Errampalli.

[f] R = Isolates which grew on 5 μg ml^{-1} or higher concentration of TBZ were determined as TBZ- resistant (TBZ^R).

[g] Not available.

[h] Isolates from AB were provided by J.D. Holley, Alberta Agriculture, Food and Rural Development, Brooks, AB.

[i] S = Isolates which failed to grow on 5 μg ml^{-1} or higher concentration of TBZ were determined as TBZ-sensitive (TBZ^S).

[j] Isolates from ND were provided by G. Secor, North Dakota State University, Fargo, ND.

Crops and Livestock Research Centre, Charlottetown PEI, and a commercial potato storage in Summerside in PEI. Twenty-seven reference isolates of *H. solani*, previously characterized as either TBZ^R or TBZ^S based on their response to TBZ on growth media (data not published), were obtained from Dr. Gary Secor, North Dakota State University, Fargo, North Dakota (ND; Table 1) and Dr. Jim Holley, Alberta Agriculture, Food and Rural Development, Brooks, Alberta (AB; Table 1).

Diagnosis and detection of *H. solani* with conventional methods

Potato tubers with silver scurf were diagnosed based on the symptomatology described by Jellis and Taylor (1977). Isolates were confirmed as *H. solani* based on the fungal identification keys of Barnett and Hunter (1998) and by a PCR assay (Errampalli *et al.* 2001b). The colonies of the fungus were assessed and identification was made based on colony morphology and color, and the morphology of conidiophores and conidia.

Conventional method for detection of TBZ^R and TBZ^S isolates of *H. solani*

All single-spore isolates of *H. solani* were tested for TBZ-sensitivity on PDA-amended with 0, 5, 10, 20, 50 μg ml^{-1} technical grade TBZ (Mertect®, Syngenta, Calgary, Canada). TBZ-resistant reference isolates, AB29-B1, AB107-B2, and AB185-A2, were grown on 0, 5, 100, 250, and 500 μg ml^{-1} of TBZ. Each isolate was grown on three plates of each of the appropriate five concentrations. Each plate was centrally inoculated with an agar block (2 mm x 2 mm), cut from the leading edge of an actively growing colony and incubated at 22 ± 2°C. The colony diam for each isolate was measured (mm) once a wk for 9 wk. The percentage of growth inhibition was calculated relative to fungicide concentration, using linear regression analysis (SigmaStat statistical software package, SPSS Science, Chicago, 1992-1995 version) and the graphs were plotted with SigmaPlot software package (SPSS Science, Chicago, 1992-1995 version). The concentration of TBZ that inhibited radial growth by 50% (EC_{50}) relative to growth on nonamended medium was determined. Isolates were classified as sensitive (TBZ^S) if they grew on nonamended agar but not on TBZ-amended culture medium, and resistant (TBZ^R) if they grew on 5-50 μg ml^{-1} of TBZ (McKay and Cooke 1997). This experiment was repeated once.

Samples of potato tubers with silver scurf

To determine the reaction of PEI isolates of *H. solani* to TBZ, the isolates and cultures of *H. solani* obtained were tested for their growth response to different concentrations of TBZ. To determine if the PCR-based assay can detect either the TBZ^R or TBZ^S isolates of *H. solani* directly from lesions of silver scurf, a portion of the silver scurf lesion from each of the tubers was used directly in the PCR-based assay. In addition, single-spore isolations made from rest of the silver scurf lesion was tested in PCR-based assay.

Soil samples

Prior to planting, a total of 16 soil samples were collected in 1999 from a research plot, which had a known history of potato planting, in PEI. One composite sample per 3.6 m x 50.0 m plot, each contained nine soil cores, was taken with a 4-cm-diam soil probe to a depth of 24 cm. The samples were air-dried in brown paper bags for 24 h and kept at 4°C until later use in DNA isolations and to prepare soil dilutions.

Soil-dilution-plate technique

Viable counts of different soil microorganisms were determined as colony forming units (cfu) g^{-1} of soil. Soil dilutions were performed by the procedure described by Errampalli *et al.* (2001b). The cfu of different soil microorganisms were recorded at 2, 3, 4, and 5 wk after incubation.

DNA extraction

The mycelium of each of the single-spore isolates of *H. solani*, grown on porous cellophane membrane (Pharmacia Biotech, QC) placed on PDA, at 20 ± 2°C for 2 wk, was removed and frozen at -20°C until further use. Genomic DNA from the mycelium (0.12 g fresh wt) or infected potato tissue (0.25 g fresh wt) was extracted by the modified Proteinase K method (Errampalli *et al.* 2001b) or a method that included a phenol:chloroform extraction (Vilgalys and Gonzalez 1990). DNA from soil samples was extracted according to Errampalli *et al.* (2001b).

Diagnosis and detection of TBZ^R or TBZ^S isolates of *H. solani* with PCR-based assay

Primers were designed from the nucleotide sequence of the 1138-bp β-tubulin gene of *H. solani* (McKay and Cooke 1997). The primers, SS-for (5' AGCATAGGCTGATGCTCGT 3') and SS-rev (5' ACCTTACCACGGAAGATACCAC 3'), that encompass the DNA that contained codons 198-200, were used to detect TBZ^R or TBZ^S *H. solani*. Mutations in these codons have been correlated with fungal resistance to benzimidazoles that included benomyl and TBZ (McKay and Cooke 1997). The primers are specific to *H. solani* as they have failed to amplify any products from the common potato pathogens tested (McKay and Cooke 1997). Amplifications were carried out in a 50 µl reaction mixture that contained 10 ng template DNA, 0.2 mM each of deoxynucleoside triphosphates, 0.2 µM of each primer, and 1 unit Taq DNA polymerase (Boeringer Manneheim, Laval, QC) in the reaction buffer supplied by the manufacturer. PCR mixtures without fungal DNA were used as negative controls. The reactions were carried out in a thermocycler (Touchgene, VWR Canlab, Mississauga, ON). The following cycling parameters were used for DNA amplifications: an initial denaturation of 5 min at 94°C; 60 s at 94°C, 30 s at 58°C, and 40 s at 72°C for 35 cycles with final extension at 72°C for 7 min. The PCR-amplified products (10 µl) were separated by electrophoresis on a 2% agarose gel in 1X TAE (40 mM Tris- acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide, and visualized under UV light (Sambrook *et al.* 1989). The experiment was repeated once. PCR assay on isolates ND24-13 and ND24-21 was performed three times.

Restriction enzyme digest

A 20 µl aliquot of PCR product was digested with 2.0 units of the restriction endonuclease enzyme, *Bsa*I (New England BioLabs, Beverly, MA) in a 40 µl reaction mixture and incubated at 50°C for 2-3 h. The results of the digest were analyzed on a 2.0% high resolution agarose gel (Bio-Rad, Mississauga, ON) and visualized as described (Sambrook *et al.* 1989).

RESULTS

Identification of TBZ^R and TBZ^S *H. solani* by conventional technique

The growth of all isolates of *H. solani* on TBZ-amended and nonamended (control) media were measured (mm). All of the 27 isolates of *H. solani* from PEI grew on 5 µg ml^{-1} or higher concentration of TBZ and were categorized as TBZ^R (Table 1). Both TBZ^R and TBZ^S were identified among the isolates of *H. solani* collected from AB and ND (Table 1). Three isolates, AB29-B1, AB107-B2, and AB185-A2, grew on 0, 5, 100, 250, and 500 µg ml^{-1} TBZ over a 5-wk period. All of the TBZ^R isolates exhibited variable response to different concentrations of TBZ. For example, two TBZ^R isolates, PHsD3b and ND24-13, grew on 5, 10, 20, and 50 µg ml^{-1} TBZ over a 5-wk period and each of the isolates exhibited variable growth response to each of the TBZ concentrations (Figs. 1A and 1B). The TBZ^S isolate, AB517-D3, grew only on nonamended medium (Fig. 1C).

Detection of TBZ^R or TBZ^S *H. solani* in field soils

The dilution-plate technique, which was used to enumerate soil inhabiting fungi, was not successful to detect *H. solani* in soils. The slow growing isolates of *H. solani* could not compete with the fast growing soil fungi such as *Alternaria* spp., *Fusarium* spp., and *Rhizoctonia solani* Kühn. Detection of *H. solani* with PCR primers, SS-for and SS-rev, from the soil samples was not successful.

Amplification of TBZ^R or TBZ^S *H. solani* from mycelium and detection in infected potato tubers by PCR and restriction digestion

Amplification of the fragment of β-tubulin was successful with fungal genomic DNA extracted with any of the two DNA extraction methods tested. The primers, SS-for and SS-rev, amplified a 872-bp target sequence of β-tubulin in genomic DNA from all of 54 isolates of *H. solani*. An annealing temperature of 58°C gave a greater yield of amplified DNA than the previously reported 54°C (data not shown). Restriction digestion of the PCR product (872 bp) with *Bsa*I

gave rise to three subfragments of 420, 390, and 62 bp in TBZ^S isolates, whereas the TBZ^R isolates generated two subfragments of sizes, 482 and 390 bp. The PCR-based assay was used to distinguish TBZ^S from TBZ^R (Table 1). All

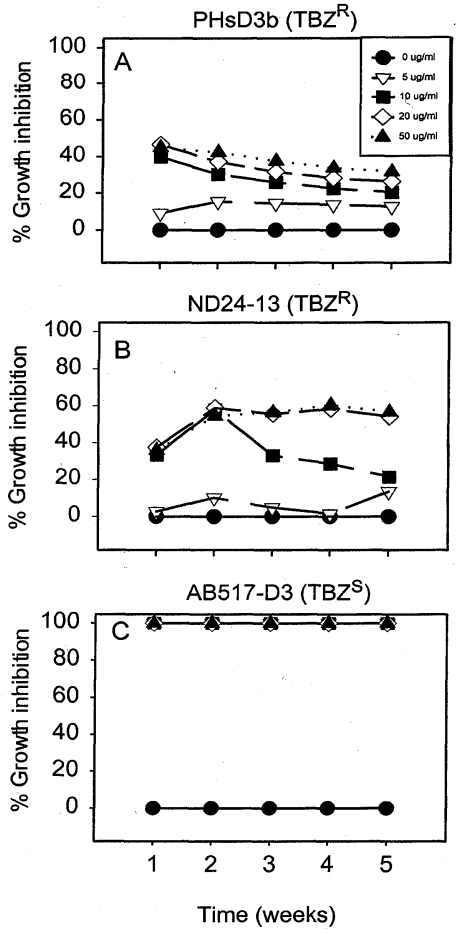

Figure 1. Reduction in *in vitro* radial growth, as a percentage of the nonamended control, of *Helminthosporium solani* isolates in response to increasing thiabendazole (TBZ) concentrations over a 5-week period. A. variable growth response to TBZ by PHsD3b (TBZ^R), an isolate collected from eastern Canada; B. variable growth response to TBZ by ND24-13 (TBZ^R), an isolate collected from ND, USA; and C. isolate AB517-D3 (TBZ^S), collected from western Canada, did not grow on four concentrations of TBZ. Each point on the graph represents the mean growth (mm) of three replicates.

of the 27 isolates of *H. solani* from PEI were TBZ^R. Of the reference isolates from AB, six were found to be TBZ^S and the other nine TBZ^R, and of the 12 reference isolates from ND, six TBZ^R and six TBZ^S were found. With the exception of ND24-13 and ND24-21, all the isolates that grew on 5 to 50 $\mu g\ ml^{-1}$ TBZ in TBZ-amended medium (data not shown) were characterized as TBZ^R with PCR-based assay (Table 1). Amplification of PCR product was not observed with common potato pathogens, *Colletotrichum coccodes* (Wallr.) S.J. Hugues (black dot), *Fusarium sambucinum* Fuckel (dry rot), *Phytophthora infestans* (Mont.) de Bary (late blight), *R. solani* (black scurf), *Verticillium dahliae* Kleb (Verticillium wilt), and *Streptomyces* spp. (common scab).

All of the 20 tuber samples infected with *H. solani* were TBZ^R. In addition, the response of the single-spore isolates of *H. solani* collected from the first half of the infected lesion on the tuber surface to TBZ correlated well with the results obtained by PCR-based assay.

The two techniques, PCR and conventional, were comparable as 52 out of 54 isolates tested gave similar results (Table 1). Only two isolates, ND24-13 and ND24-21, determined as TBZ^R with conventional technique, were identified as TBZ^S with PCR-based assay (Table 1). To reconfirm the results on these two isolates, both isolates were tested twice and similar results were obtained.

DISCUSSION

The PCR-based assay described to identify either TBZ^R or TBZ^S isolates of *H. solani* that infects potatoes is more rapid, and highly sensitive compared to the conventional method of TBZ^R identification. An identically-sized product was formed when primers, SS-for and SS-rev, were used in a PCR test with purified DNA from mycelium and *H. solani* infected potato tissue. TBZ^R or TBZ^S isolates of *H. solani* were distinguished by the use of a restriction digest with *Bsa*I, that produced fragments

of different sizes. The PCR primers were very specific to isolates of *H. solani*, as an amplification product was generated with TBZ-sensitive or -resistant isolates of *H. solani* but not with some of the most common potato pathogens, such as *C. coccodes*, *F. sambucinum*, *P. infestans*, *R. solani*, *V. dahliae*, or with potato DNA. Similarly, the PCR-based assay on common pathogens of potato failed to produce amplification product (McKay and Cooke 1997).

In this study, we have shown that a PCR-based assay can be used to distinguish between TBZ^R and TBZ^S in 54 isolates of *H. solani* collected in North America from three different geographical regions separated by at least 2000 km. With the exception of a report from the UK, in which PCR-based assay was used to detect TBZ^R-*H. solani* (McKay and Cooke 1997), traditionally, thiabendazole resistance in isolates of *H. solani* has been detected by observing the growth response of isolates on TBZ-amended media (Hide *et al.* 1988; Holley and Kawchuck 1996; Platt 1997). Both PCR-based assay and conventional method are concordant with each other for 96% of isolates (Table 1). A discrepancy between results produced by the two methods was observed in only two isolates, ND24-13 and ND24-21. These isolates were determined as TBZ^R with the conventional method, but the results from the PCR-based assay showed them as TBZ^S. There may have been a mix-up of more than one conidia in the initial isolations (McKay and Cooke 1997). Another reason could be that a DNA mutation other than the one that is detected by the PCR-based assay may be responsible for the TBZ-resistance exhibited by ND24-13 and ND24-21 on TBZ-amended medium.

In this study, isolates of *H. solani* gave a variable growth response on > 5.0 μg ml^{-1} of TBZ. However, except ND24-13 and ND24-21, all the isolates that grew on TBZ-amended medium were confirmed as TBZ^R with the PCR-based study. Variable growth responses were previously reported and we believe that the differences in sensitivity to TBZ among isolates may be conferred by more than one mutation (McKay and Cooke 1997; Platt 1997). Further studies are needed to determine if the isolates of *H. solani* used in this study, with variable growth response, have multiple β-tubulin mutations.

All the isolates of *H. solani* collected from PEI were TBZ^R. The isolates were obtained from tubers collected from commercial warehouse, where the application of TBZ was ineffective in inhibiting the development of silver scurf in previous years. We confirmed that the increase in silver scurf on tubers, in the specific warehouse used in this study, was due to the development of resistance of isolates of *H. solani* to TBZ. Similarly, TBZ^R *H. solani* was isolated from potatoes stored in a warehouse that received repeated applications of TBZ (Hide *et al.* 1988; Platt 1997).

The detection of *H. solani* in soil samples from a field, where potatoes with silver scurf were harvested, was not successful. Due to the competition from fast growing soil fungi such as *Fusarium* spp., *Alternaria* spp., and *R. solani*, the slow growing *H. solani* could not be isolated with the dilution-plate technique. PCR-based assay, with primers SS-for and SS-rev, was also unsuccessful to detect TBZ-resistant or -sensitive isolates of *H. solani* from soil. This is probably from the variability of ratio of target DNA to non-target DNA extracted from soils. Similarly, *H. solani* could not be detected with a first round of PCR, but a nested PCR, that utilized amplified ITS amplicons from the first round of PCR as a template, with species-specific nested primers from the ITS region was successful to detect isolates of *H. solani* from the same soil samples (Errampalli *et al.* 2001b). The nested PCR utilizes the first PCR product as a template and amplifies the product. To detect low levels of inoculum of TBZ-resistant or -sensitive *H. solani* from soil, further studies are needed to develop a more sensitive PCR detection method.

The advantage of PCR-based assay over the conventional method of TBZ^R identification is its rapidity. All the potato tubers with silver scurf collected from commercial store for this study were tested for TBZ reaction in 1 d using

the PCR-based assay, whereas it took 5 wk to obtain the same information with the conventional method. The PCR-based assay can detect either TBZ^R or TBZ^S directly from tuber tissue within 1 d. The PCR-based assay provides rapid identification of the TBZ-resistance in the pathogen and thereby allows for appropriate and timely implementation of strategies to manage silver scurf disease. An integrated disease management strategy for long term control of silver scurf may include cultural, biological, and chemical control methods, in field and in storage (Errampalli *et al.* 2001a; Secor and Gudmestad 1999). Both mixtures and alternations with non-benzimidazole fungicides are acceptable methods to prevent and to manage resistance to benzimidazoles (Delp 1988). Some of the seed tuber treatments effective against silver scurf include mancozeb and fludioxonil (Cayley *et al.* 1983; Frazier *et al.* 1998; Secor and Gudmestad 1999). Integration of cultural methods such as crop rotation, prevention of tuber bruising, in combination with chemical seed treatment at planting and after harvest, and proper management of potato stores can help reduce silver scurf (Errampalli *et al.* 2001a).

ACKNOWLEDGEMENTS

D. Errampalli conducted research on silver scurf of potato at the Crops and Livestock Research Centre (CLRC), Agriculture and Agri-Food Canada, Charlottetown, P.E.I. Janet Saunders' B.Sc honours thesis project on "the PCR detection of TBZ^R *H. solani* collected from tubers in PEI" was conducted under the supervision of D. Errampalli at CLRC. The authors wish to thank Dr. A. Svircev, SCPFRC, for her comments on this paper.

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