RAPID DETECTION OF BENZIMIDAZOLE RESISTANCE IN *RHYNCHOSPORIUM SECALIS* USING ALLELE-SPECIFIC OLIGONUCLEOTIDE PROBES.

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ABSTRACT

Benzimidazole fungicides are important mixture components in strategies to combat fungicide resistance in *Rhynchosporium secalis*. These strategies have recently been threatened by the detection of benzimidazole resistance in field strains of *R. secalis*. To evaluate the significance of this resistance, and monitor its spread, a rapid and accurate method for identifying resistant alleles has been developed. Hybridisation of allele-specific oligonucleotide (ASO) probes to Polymerase Chain Reaction(PCR) amplified β-tubulin gene fragments has successfully detected single DNA base-pair mutations in several Rhynchosporium strains. Combining ASO detection with sequencing of β-tubulin gene fragments only one point mutation, substituting glycine for glutamic acid at codon 198, occurred in benzimidazole-resistant field populations. Two probes, one sensitive and one resistant, were sufficient to monitor current field populations. A second mutation substituting lysine for glutamic acid, which was generated in the laboratory, has not so far been detected in field populations. This technology should improve efforts to evaluate anti-resistance strategies in practice.

INTRODUCTION

Barley leaf blotch, caused by *Rhynchosporium secalis* (Oudem) J. J. Davis, is an important disease in the cool maritime regions of the world. It commonly causes a 2-5% yield reduction but, in severe epidemics losses can reach 40% (Jenkins & Jemmett, 1967). It is a difficult wet weather disease to control, particularly when spraying is delayed and disease thresholds are missed. Resistant cultivars have not been durable because of rapid selection of novel races from the varied gene pool, and fungicides play a major role in control measures. Fungicides that inhibit the C-14 demethylation step in sterol biosynthesis (DMIs) have been very successful in controlling the disease, but efficacy is now being eroded by the development of resistance to some members of this group (Kendall *et al.*, 1993)

Monitoring *R. secalis* populations throughout the U.K. between 1980 and 1989 failed to detect strains resistant to benzimidazoles (benomyl, carbendazim, thiophanate methyl). Consequently, mixtures of benzimidazoles and DMI fungicides were recommended for leaf blotch control in an effort to combat further spread of resistance to DMIs (Hollomon, 1992). In 1990, the first benzimidazole-resistant strains were detected in the U.K. at an incidence of 1% (Hollomon & Kendall, 1993). Since then, U.K. wide monitoring has revealed frequencies of benzimidazole resistance in field populations ranging from 1% in

parts of Northern England (Phillips & Locke, 1994), to over 50% in N.Ireland (Taggart *et al.*, 1994).

It is important to monitor for the development and spread of benzimidazole resistance so that strategies can be evaluated, and advice changed if necessary. Unfortunately, current methods to detect resistance based on bioassay are of limited value. Resistant mutants can only be detected when their frequency reaches 1% or higher in the population, and the methodology to achieve this is both labour-intensive and time-consuming. Isolation of pure cultures of *R. secalis* from infected field samples can take up to 6 weeks, with isolation becoming increasingly difficult as the season progresses. Conclusive results obtained by *in vitro* bioassay can take a further 2 weeks, but this fails to distinguish between different benzimidazole-resistant alleles. It has previously been shown that benzimidazole resistance in *R. secalis* is correlated with decreased binding of these fungicides to tubulin-like proteins (Kendall, *et al*, 1994). To improve efficiency of monitoring procedures, we are seeking to develop a more rapid detection technique. In this paper, the successful application of allele-specific oligonucleotide probes to detect benzimidazole resistance caused by a single base change at codon 198 in the β-tubulin gene is reported.

METHODS

Isolation and bioassay of *R. secalis*.

Single spore isolates were obtained from diseased leaves using methods fully described elsewhere (Hollomon, 1984), and assayed in 25-well repli-plates on Czapek dox agar with 0.5% mycological peptone (CDM) according to procedures given in Kendall, *et al.*, (1993). Carbendazim was a gift from BASF, Limburgerhof, Germany, and diethofencarb was supplied by Sumitomo Chemical Company, Takarazuka, Japan. Both fungicides were technical grade.

DNA preparation, amplification and sequencing.

DNA was prepared from freeze-dried cells (10mg) by vortexing with glass beads in 600μl buffer (lysis) and extraction with phenol/chloroform. DNA was precipitated from the aqueous phase with ethanol, resuspended in water and treated with RNAase. This DNA was used directly as template for both PCR amplification using two 24-mer oligonucleotide primers designed to produce a 416 bp fragment encompassing codon 198 of the *R. secalis* β-tubulin gene, and sequenced. PCR(100μl) containing primers, template DNA, nucleotides and Taq-polymerase were carried out in an Autogene temperature cycler (Grant Instruments, Cambridge, UK.) and involved an initial five minute denaturation step followed by 30 cycles of anealing at 65°C for one minute, extension at 72°C for one minute and denaturation at 94°C for one minute. Production of the target DNA was checked by electrophoresis on 3% Nusieve agarose gel. This product was used directly in the ASO probe assay or was sequenced after purification.

Allele-specific oligonucleotide (ASO) probe detection

The PCR generated β-tubulin gene fragment was dot-blotted onto either a nylon membrane for radioactive (^{32}P) detection, or onto a nitro-cellulose membrane where a non-

radioactive labelling system was used. Fifteen-mer oligonucieotide probes designed to match the expected sequence surrounding codon 198 were end-labelled with either ^{32}P or biotin, and hybridised to target DNA using standard procedures. Control of washing temperature allowed mismatched probes to be washed away. Hybridised, and correctly aligned, ASO probes were visualised either by radioautography, or by a non-radioactive detection system (Boehringer Mannheim) which uses streptavidin/alkaline phosphatase and nitro-blue tetrazolium salt as the substrate. Figure 1 shows the full procedure used to detect these single DNA base changes.

Results

Sequencing an amplified fragment of the β-tubulin gene of *R. secalis* has linked benzimidazole resistance with a single DNA base change at codon 198. Table 1 shows results for five strains representing the different sequences so far encountered. In all resistant strains so far isolated from field crops, adenine is replaced by guanine causing the substitution of glycine for glutamic acid at amino acid 198. These strains always show negativ cross-resistance between carbendazim and diethofencarb. In a u.v. irradiated resistant mutant (BEN 22), derived from the wild-type strain K1124, replacement of a guanine in codon 198 with adenine causes substitution of lysine for glutamic acid.

ASO probe detection: Effects of washing temperature on stringency.

Stringency of the washing temperature required to remove any mismatched probe was determined by the probe being used. No selectivity was observed at 40°C, and probes bound to DNA fragments amplified from benzimidazole-resistant and -sensitive strains alike. At 48°C, all mismatched sensitive ASO probe was washed away from DNA of resistant strains following three two-minute washings, allowing specific detection of sensitive strains. For an ASO specific for the resistant mutation GAG--GGG, it was necessary to raise the washing temperature to 50°C in order to selectively detect this mutation. This same probe did not detect the resistant mutation GAG--AAG when membranes were washed at 50°C. So far we have not detected this resistant mutation in field strains. This procedure has correctly identified the resistance status of all strains for which we have bioassay and sequence data.

Discussion

Single base-pair mutations in *R. secalis* causing amino acid substitutions and correlated with benzimidazole resistance, were similar to those found in resistant field strains of other plant pathogenic fungi (Koenraadt & Jones, 1992; Martin, et al, 1992; Yarden & Katan, 1993). In both *R. secalis*, *Botrytis cinerea* (Yarden & Katan, 1993), and *Venturia inaequalis* (Koenraadt, *et al*, 1992) substitution of a lysine for glutamic acid results in a loss of the negatively correlated cross-resistance between benzimidazoles and diethofencarb, although this mutation has yet to be found in field populations of *Rhynchosporium*.

ASO probes provide a rapid diagnostic technique which takes less than 24h to complete. At present, the method requires DNA template extracted from pure cultures of *R. secalis*, but we are currently exploring nested primer PCR approaches for direct

detection from diseased leaves. This should reduce the time taken to obtain a result from field samples to 24-48h, compared with 6-8 weeks now required for conventional isolation and bioassay. This technology not only provides an accurate detection of resistance but can distinguish between different resistant alleles. Although up to 10 sites within the β-tubulin gene have been identified as conferring benzimidazole resistance (Osmani & Oakley, 1991), it is clear that mutations in field strains of phytopathogens are restricted to codons 198 and 200 (Koenraadt & Jones, 1992; Adachi, *et al*, 1993; Yarden & Katan, 1993). Consequently, the number of different ASO probes needed to monitor field populations is quite limited; only four probes were needed to accurately identify benzimidazole resistance in a large world-wide collection of *V. inaequalis* strains (Koenraadt & Jones, 1992). For obligate or slow growing pathogens rapid detection of resistance using ASO probes should allow more samples to be tested and so contribute to more effective monitoring of the performance of strategies used to combat resistance. Where efforts to assess the risk of resistance to new compounds identifies resistant mutants at an early stage in development, rapid, DNA based, detection offers a more effective way to identify rare mutants in field populations, than conventional monitoring methods.

Acknowledgements

We thank MAFF and H-GCA for funding this work.

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FIGURE 1. Allele-specific oligonucleotide DNA probes

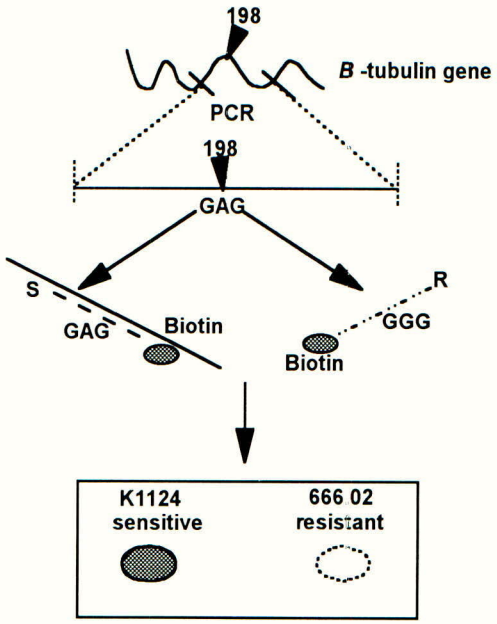

TABLE 1. DNA changes at codon 198 in the *B*-tubulin, and benzimidazole resistance in *Rhynchosporium secalis*..

Isolate	Amino acids						Fungicide sensitivity	
	196 SER	197 ASP	198 GLU	199 THR	200 PHE	201 CYS	Benzimidazoles	N-phenyl-carbamate
K1124	TCT	GAT	*GAG*	ACC	TTC	TGT	*S*	*R*
809.02	TCT	GAT	*GAG*	ACC	TTC	TGT	*S*	*R*
666.02	TCT	GAT	GLY *GGG*	ACC	TTC	TGT	*R*	*S*
769.03.03	TCT	GAT	*GGG*	ACC	TTC	TGT	*R*	*S*
BEN22	TCT	GAT	LYS *AAG*	ACC	TTC	TGT	*R*	*R*

MUTATIONS IN THE BETA-TUBULIN GENE OF BENOMYL-RESISTANT PHENOTYPES OF *BOTRYTIS CINEREA*

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Three phenotypes were identified among benomyl-resistant strains of *Botrytis cinerea* in Israel, when tested for sensitivity to carbendazim (MBC) and diethofencarb (NPC): $Ben^{HR}NPC^{S}$= highly resistant to MBC (EC_{50}>50 µg/ml) and sensitive to 0.5µg/ml NPC; $Ben^{MR}NPC^{R}$= moderately resistant to MBC ($10 \leq EC_{50} < 20$ µg/ml) and resistant to 10 µg/ml NPC; and $Ben^{HR}NPC^{R}$= highly resistant to MBC and resistant to NPC. A 1-kb fragment of the wild-type gene encoding for beta-tubulin (designated *benA*) in *B. cinerea* was cloned and sequenced. The deduced partial amino acid sequence of the *B. cinerea* beta-tubulin showed a high degree of similarity to beta-tubulins of other filamentous fungi. A PCR approach was used to amplify and sequence 992-bp *benA* fragments from strains representing the three phenotypes. In the eight Ben^{R} strains analyzed, three single base-pair mutations were identified and found to correlate with the different phenotypes: codon 198, encoding glutamic acid in the wild type, was changed to an alanine codon in the $Ben^{HR}NPC^{S}$ phenotype, or to a lysine codon in the $Ben^{HR}NPC^{R}$ phenotype; codon 200, encoding phenylalanine was changed to a tyrosine codon in the $Ben^{MR}NPC^{R}$ phenotype. These mutations were similar to those identified in benomyl-resistant field strains of other phytopathogenic fungi.

RESISTANCE OF *BOTRYTIS CINEREA* TO DICARBOXIMIDES, BENZIMIDAZOLES AND PHENYLCARBAMATES IN THE CHAMPAGNE VINEYARDS

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ABSTRACT

Strains of *B. cinerea* resistant either to dicarboximides (e.g. iprodione, procymidone, vinclozolin) or to both benzimidazoles (e.g. carbendazim) and phenylcarbamates (e.g. diethofencarb) are commonly found in Champagne vineyards. Limitations in the use of these fungicides are recognized as anti-resistance strategies. However, when the frequencies of resistant strains are too high, they can be temporarily withdrawn. Such advice is given according to the results of the monitoring done every year at vintage.

INTRODUCTION

In french vineyards, *B. cinerea* remains a parasite feared by vine-growers because of its qualitative and quantitative effects on wine production. The chemical control of this fungus is normaly achieved by three of four treatments applied between the flowering stage and three weeks before vintage. Several families of fungicides are registered in France. The oldest ones are protectants (e.g. chorothalonil, dichlofluanid, folpet, thiram) and have never been affected by resistance because of their multi-site effects. On the other hand, resistance developed towards dicarboximides (e.g. clozolinate, iprodione, procymidone, vinclozolin), benzimidazoles (e.g. benomyl, carbendazim, thiophanate-methyl) and phenylcarbamates (e.g. diethofencarb). The evolution of such phenomena in Champagne vineyards over 10 years and the anti-resistance strategies will be presented in this paper.

CHARACTERISTICS OF THE VARIOUS RESISTANT STRAINS

Dicarboximides are among the most effective fungicides against *B. cinerea*. However the development of moderately resistant strains (Rd ; Table 1) provided inadequate control of grey mould in Champagne vineyards and also in many other French regions (Leroux and Clerjeau, 1985).

Benzimidazoles which were introduced before dicarboximides, selected highly-resistant strains which were very susceptible to the phenylcarbamate diethofencarb (Rb1 ; Table 1). Since the commercialization of mixtures diethofencarb/carbendazim in 1987, a new phenotype simultaneously-resistant to these two fungicides appeared (Rb2 : Table 1).

All strains remained sensitive to pyrimethanil, an anilinopyrimidine recently introduced in France (Table 1).

TABLE 1 : Effects of fungicides on the germ-tube elongation of various strains of B. cinerea[a] collected in Champagne vineyards in 1992 and 1993

Fungicides	Mean EC50 (mg/l) of phenotypes				
	Sd	Rd	Sb	Rb1	Rb2
iprodione	0.8	5.0	-	-	-
procymidone	0.6	8.0	-	-	-
vinclozolin	0.4	5.0	-	-	-
carbendazim	-	-	0.05	>25	5.0
diethofencarb	-	-	>25	0.05	>25
pyrimethanil	0.07	0.07	0.06	0.07	0.07

[a] : the tests were conducted according to the method of Leroux and Gredt (1989) on four to eight strains of each type.

TABLE 2 : Relationship between the percentages of B. cinerea resistant strains and the efficacies of a programme combining several fungicides (average values obtained in 3 or 4 trials each year)

years	Control plots		Treated plots[a]
	% Rd	% Rb2	% efficacy
1989	25	1	50
1990	31	14	72
1991	38	34	13
1992	20	33	17

[a] : the programme consisted in three applications of a mixture carbendazim/diethofencarb (500 + 500 g/ha) followed by a mixture vinclozolin/thiram (500 + 3200 g/ha) and a dicarboximide alone (procymidone or vinclozolin at 750 g/ha).

RESISTANCE PHENOMENA IN FIELD TRIALS

From the various trials conducted in Champagne region over the last six years, it has been shown that :

- the selection pressure induced by one application of procymidone or vinclozolin (750 g/ha) towards Rd strains was lower than that of a mixture of carbendazim/diethofencarb (500 + 500 g/ha) towards Rb2 strains (Leroux and Moncomble, 1993).

- at reduced rates (500 g/ha instead of 750 g/ha) procymidone and vinclozolin used in mixture with thiram (3200 g/ha) exerted a selection pressure significantly lower than that of dicarboximides applied alone at their full rate (750 g/ha) (Leroux and Moncomble, 1993).

- the programme based on three applications of a mixture carbendazim/diethofencarb, followed by a mixture vinchlozolin/thiram, and a dicarboximide alone was effective in 1989 and 1990 but failed to control grey mould in 1991 and 1992. It seemed that this was mainly due to the development of Rb2 strains (Table 2).

MONITORING AND RECOMMENDATIONS OF FUNGICIDE USE

A monitoring of resistance of B. cinerea is conducted every year in more than 100 commercial Champagne vineyards at vintage. In each location, 10 to 20 diseased berries are collected and the percentages of the various phenotypes are determined according to the method of Leroux and Clerjeau (1985).

TABLE 3 : Evolution of number of annual fungicide treatments and percentages of B. cinerea resistant strains in Champagne vineyards between 1982 and 1993

years	dicarboximides		carbendazim and diethofencarb		
	Tre.[a]	% Rd	Tre.[a]	% Rb1	% Rb2
1982	4	87	0	85	0
1983	0	72	0	82	0
1984	0	42	0	76	0
1985	0	22	0	94	0
1986	0.5	21	0	97	0
1987	0.8	30	0.8	97	0
1988	0.8	22	1.5	95	2
1989	1.1	30	1.1	77	21
1990	1.6	37	0.9	52	46
1991	1.7	48	0.9	38	62
1992	1.5	56	0.2	54	46
1993	0	34	0	70	29

[a] : average number of annual treatments with dicarboximides (alone or in mixture with thiram) or mixtures carbendazim/ diethofencarb.

From the results of Table 3, it appeared that the frequencies of dicarboximide-resistant strains (Rd) as well as those of strains doubly resistant to carbendazim and diethofencarb (Rb2) decreased when the selection pressure ceased (this was not the case with the Rb1 strains). This non- persistant resistance which is probably due to reduced fitness of Rd and Rb2 strains permitted a discontinious use of both dicarboximides and mixtures carbendazim/diethofencarb. The re-employment of these fungicides occured when the average percentages of resistant strains were below 25 % at the preceeding vintage.

This survey confirmed than one annual application of dicarboximides (alone or in mixture with thiram) exerted lower selection pressure than that of mixtures carbendazim/diethofencarb (Table 3).

CONCLUSION

In spite of the limitation of annual treatments against B. cinerea (three instead of four or five in the 1980's) and of combined uses of the varicus available fungicides (mixtures or alternations), the chemical control of grey mould remains uncertain in Champagne vineyards. As an example, the recommendation for 1994, advises the successive applications of a mixture carbendazim/diethofencarb, of pyrimithanil and of a dicarboximide (alone or in mixture with thiram). This may lead to selection of RB2 strains and possibly Rd ones, which is likely to lead (once more) to the withdrawal of mixtures carbendazim/diethofencarb and dicarboximides in 1995. Other considerations, such as residue levels or negative effects on fermentation are additional constraints for the establishing of grey mould treatment programmes.

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DISTRIBUTION AND INCIDENCE OF BENZIMIDAZOLE-RESISTANT FUSARIUM SAMBUCINUM AND HELMINTHOSPORIUM SOLANI ISOLATED FROM POTATO IN NORTH AMERICA.

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ABSTRACT

Isolates of Fusarium sambucinum and Helminthosporium solani were recovered from dry rot and silver scurf diseased potatoes collected from states and provinces throughout the United States, Mexico, and Canada. Single spore cultures of both fungi were prepared and tested for resistance to thiabendazole (TBZ) by growth reduction (ED50) on TBZ-amended acid PDA (Fusarium) or modified V-8 (H. solani) media. Of 504 Fusarium isolates representing 18 states, 378 or 75% of them, were resistant to TBZ. The resistance ranged from 1 to 64 ppm. The Fusarium isolated was almost exclusively F. sambucinum. Of the H. solani isolates tested representing 7 states, 21/27 or 77% were resistant to TBZ. The resistance ranged from 20 to 50 mg/l. TBZ-resistant isolates were cross-resistant to thiophanate-methyl.

INTRODUCTION

Storage diseases of potato can cause serious problems costing producers and processors large amounts of time and money. Many of these diseases can be controlled by post-harvest application of fungicides as the potatoes are going into storage. The only post harvest chemical approved for potatoes in the US is thiabendazole ('Mertect 340F'). This chemical has been intensively used and has provided excellent primary control of dry rot, caused by Fusarium sambucinum (Fs), for 20 years, and assumed control of silver scurf, caused by Helminthosporium solani (Hs), even though it is not a target disease for this fungicide. Recent outbreaks of both dry rot and silver scurf prompted us to re-examine these diseases, and the role that fungicide application has on disease management, including possible fungicide resistance to TBZ.

METHODS AND MATERIALS

Isolations

Fusarium was isolated from dry rot diseased potato samples of various cultivars collected from throughout the US, Mexico, and Canada. Most samples were sent by colleagues at universities and industry. Fusarium was successfully isolated from samples representing 18 states or provinces (Table 1). Potatoes were surface sterilized (0.5% Na hypochlorite, 15 min) and small pieces of tuber tissue at the margin of the dry rot lesion placed on acidified potato dextrose agar (APDA). After 5-7 days incubation, Fusarium cultures were transferred to PDA medium. Single spore cultures of each isolate were

prepared. 504 Fs cultures were collected for testing (Table 1). Carnation leaf agar was used to identify isolates (Nelson et al, 1983).

Hs was isolated from naturally-infected silver scurf tubers recovered from samples collected or sent from 7 states or provinces (Table 2). Surface sterilized tubers, (0.5% Na hypochlorite, 15 min), were incubated in a humid chamber at room temperature. After 5-7 days, conidia were picked from the tuber surface and spread on APDA. After 24-58 hrs, germinated single spores were transferred to modified V-8 agar medium.

Resistance testing

Single spore Fs colonies were tested for TBZ resistance using PDA amended with technical grade TBZ (98.5%), provided by Merck & Co., Rahway, NJ, at 0, 10, 20, 50 and 100 mg/l. Mycelial disks 5 mm in diameter were removed from Fs cultures 5-7 days old, and a single disk was centered in petri plates with or without TBZ. Cultures were incubated at room temperature (approx 25°C) for 7 days under continuous fluorescent light. Each treatment was replicated three times; the experiment was repeated twice.

Single spore Hs colonies were tested for TBZ resistance using modified V-8 (MV8) medium amended by the addition of technical grade TBZ as above. TBZ concentrations ranged from 0 to 200 ppm. Single mycelial disks 5 mm in diameter were removed from the edge of one-month-old cultures and centered in petri dishes with or without TBZ. Cultures were incubated 21 days at 23°C in the dark. Each treatment was replicated four times; the experiment was repeated twice.

Calculation of TBZ resistance

Resistance data was based on the inhibition of radial mycelial growth of the colony after 7 (Fs) or 21 (Hs) days. Two diameter measurements were taken for each colony at perpendicular axes to each other and averaged. The logarithm of the concentration versus the logarithm of the percentage growth reduction was plotted and regression analysis used to determine graphically the concentration in mg/l of the TBZ causing 50% reduction of colony growth compared to the unamended control; this was defined as ED50 (Trivellas, 1988).

RESULTS

Of the Fs isolates recoverd from potato tubers with dry rot, greater than 95% were identified as Fusarium sambucinum. The results of TBZ resistance testing of these isolates can be seen in Table 1. Overall, 378/504 isolates tested, or 75%, were resistant to TBZ. The ED50 ranged from 1-64 mg/l.

The results of resistance testing with Hs isolates can be seen in Table 2. Of the isolates of Hs recovered from silver scurf affected tubers, 21/27, or 77%, were resistant to TBZ. Resistance ranged from 20-50 mg/l.

CONCLUSION

It is evident that resistance to TBZ is geographically widespread in fungi causing the post- harvest diseases dry rot and silver scurf in North America. This data should be used in formulating management strategies for control of these diseases.

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TABLE 1. Distribution and frequency of TBZ-resistant Fusarium[1] isolates in North America

Isolate Source	Number Tested	Percent Sensitive	Percent Resistant	ED50[2]
North Dakota	76	18	82	30
Minnesota	30	30	70	21
South Dakota	2	100	0	< 10
Idaho	255	16	84	27
Wisconsin	9	78	22	25
Maine	4	100	0	< 10
Texas	1	100	0	< 10
Washington	7	100	0	< 10
Alaska	4	75	25	53
Colorado	15	93	7	21
New York	39	3	97	29
Michigan	17	0	100	33
Nebraska	12	25	75	32
Alabama	4	100	0	< 10
Utah	5	0	100	31
Delaware	2	100	0	< 10
Wyoming	7	29	71	29
Pr. Ed. Is., Canada	4	100	0	< 10
Michoacan, Mexico	12	66	33	13
Total	504	25	75	

[1]Almost exclusively F. sambucinum

[2]Estimated using colony diameter reduction after seven days

TABLE 2. Frequency and distribution of TBZ-resistant Helminthosporium solani isolates from North America

Isolate Source	Number Tested	Percent Sensitive	Percent Resistant	ED50[1]
North Dakota	14	7	93	20-47
Minnesota	2	50	50	50
Wisconsin	2	100	0	--
Alaska	1	100	0	--
Oregon	1	100	0	--
New Brunswick	5	0	100	25-32
Maine	2	0	100	20-32
Total	27	22	78	

[1]Estimated using colony diameter reduction after 21 days

IMPACT OF BENZIMIDAZOLE-RESISTANT FUSARIUM SAMBUCINUM ON DRY ROT DISEASE OF STORED POTATOES.

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ABSTRACT

Isolates of Fusarium sambucinum (Fs) with high, medium, and low resistance to thiabendazole (TBZ) or susceptible were evaluated for their impact on dry rot incidence and severity of inoculated potatoes. Potato tubers, cv. Norchip, were mechanically bruised, inoculated with Fs spores, treated with TBZ by dip or low-volume spray, and evaluated after 2,3,4 and 5 months' storage at 10°C. The experiment was conducted twice. In most instances, TBZ resistance resulted in increased incidence and severity of dry rot compared to the control treatments. Method of TBZ application did not appreciably affect the incidence or severity of dry rot in stored potato tubers.

INTRODUCTION

Previous work (Secor et al., these proceedings) has shown widespread resistance of Fusarium sambucinum (Fs) to the fungicide thiabendazole (TBZ). Of 504 isolates of Fs tested, 378, or 75% of them were resistant. The ED50 ranged from 1 to 64 mg/l. TBZ is the only fungicide registered for post-harvest application and has specific activity against Fusarium on a number of crops. Dry rot disease has recently become a serious problem of stored potatoes after twenty years of excellent control which coincides with the widespread use of TBZ. Because fungicide resistance is usually irreversible, it was of interest to determine if the resistance observed has an impact on the ability of TBZ to control dry rot. Similar studies (Hall and Hide, 1992) have shown that resistance to TBZ in Helminthosporium solani does reduce the ability of post-harvest application of TBZ to control silver scurf. The purpose of these studies was to determine the role TBZ resistance in Fs may play in the development of dry rot disease in stored potatoes.

METHODS AND MATERIALS

Potato tubers, cv. Norchip, were used in all studies. In the first year of the study, potato tubers were inoculated with one of eight isolates of Fs that varied in their response to TBZ and could be categorized as susceptible (<10 mg/l) or resistant to low (15-20 mg/l), medium (25-30 mg/l) or high (>40 mg/l) levels of the fungicide. Four isolates of each TBZ response category were recovered from dry rot infected tubers originating from North Dakota and four were from Idaho.

Potato tubers were bruised in a small batch cement mixer for 60 seconds prior to inoculation. Fusarium cultures used for inoculum were grown on PDA at room temperature under continuous fluorescent light. Inoculum was prepared from 5-7 day old single spore

parts of Northern England (Phillips & Locke, 1994), to over 50% in N.Ireland (Taggart *et al.*, 1994).

It is important to monitor for the development and spread of benzimidazole resistance so that strategies can be evaluated, and advice changed if necessary. Unfortunately, current methods to detect resistance based on bioassay are of limited value. Resistant mutants can only be detected when their frequency reaches 1% or higher in the population, and the methodology to achieve this is both labour-intensive and time-consuming. Isolation of pure cultures of *R. secalis* from infected field samples can take up to 6 weeks, with isolation becoming increasingly difficult as the season progresses. Conclusive results obtained by *in vitro* bioassay can take a further 2 weeks, but this fails to distinguish between different benzimidazole-resistant alleles. It has previously been shown that benzimidazole resistance in *R. secalis* is correlated with decreased binding of these fungicides to tubulin-like proteins (Kendall, *et al*, 1994). To improve efficiency of monitoring procedures, we are seeking to develop a more rapid detection technique. In this paper, the successful application of allele-specific oligonucleotide probes to detect benzimidazole resistance caused by a single base change at codon 198 in the β-tubulin gene is reported.

METHODS

Isolation and bioassay of *R. secalis*.

Single spore isolates were obtained from diseased leaves using methods fully described elsewhere (Hollomon, 1984), and assayed in 25-well repli-plates on Czapek dox agar with 0.5% mycological peptone (CDM) according to procedures given in Kendall, *et al.*, (1993). Carbendazim was a gift from BASF, Limburgerhof, Germany, and diethofencarb was supplied by Sumitomo Chemical Company, Takarazuka, Japan. Both fungicides were technical grade.

DNA preparation, amplification and sequencing.

DNA was prepared from freeze-dried cells (10mg) by vortexing with glass beads in 600μl buffer (lysis) and extraction with phenol/chloroform. DNA was precipitated from the aqueous phase with ethanol, resuspended in water and treated with RNAase. This DNA was used directly as template for both PCR amplification using two 24-mer oligonucleotide primers designed to produce a 416 bp fragment encompassing codon 198 of the *R. secalis* β-tubulin gene, and sequenced. PCR(100μl) containing primers, template DNA, nucleotides and Taq-polymerase were carried out in an Autogene temperature cycler (Grant Instruments, Cambridge, UK.) and involved an initial five minute denaturation step followed by 30 cycles of anealing at 65°C for one minute, extension at 72°C for one minute and denaturation at 94°C for one minute. Production of the target DNA was checked by electrophoresis on 3% Nusieve agarose gel. This product was used directly in the ASO probe assay or was sequenced after purification.

Allele-specific oligonucleotide (ASO) probe detection

The PCR generated β-tubulin gene fragment was dot-blotted onto either a nylon membrane for radioactive (^{32}P) detection, or onto a nitro-cellulose membrane where a non-

radioactive labelling system was used. Fifteen-mer oligonucieotide probes designed to match the expected sequence surrounding codon 198 were end-labelled with either ^{32}P or biotin, and hybridised to target DNA using standard procedures. Control of washing temperature allowed mismatched probes to be washed away. Hybridised, and correctly aligned, ASO probes were visualised either by radioautography, or by a non-radioactive detection system (Boehringer Mannheim) which uses streptavidin/alkaline phosphatase and nitro-blue tetrazolium salt as the substrate. Figure 1 shows the full procedure used to detect these single DNA base changes.

Results

Sequencing an amplified fragment of the β-tubulin gene of *R. secalis* has linked benzimidazole resistance with a single DNA base change at codon 198. Table 1 shows results for five strains representing the different sequences so far encountered. In all resistant strains so far isolated from field crops, adenine is replaced by guanine causing the substitution of glycine for glutamic acid at amino acid 198. These strains always show negativ cross-resistance between carbendazim and diethofencarb. In a u.v. irradiated resistant mutant (BEN 22), derived from the wild-type strain K1124, replacement of a guanine in codon 198 with adenine causes substitution of lysine for glutamic acid.

ASO probe detection: Effects of washing temperature on stringency.

Stringency of the washing temperature required to remove any mismatched probe was determined by the probe being used. No selectivity was observed at 40°C, and probes bound to DNA fragments amplified from benzimidazole-resistant and -sensitive strains alike. At 48°C, all mismatched sensitive ASO probe was washed away from DNA of resistant strains following three two-minute washings, allowing specific detection of sensitive strains. For an ASO specific for the resistant mutation GAG--GGG, it was necessary to raise the washing temperature to 50°C in order to selectively detect this mutation. This same probe did not detect the resistant mutation GAG--AAG when membranes were washed at 50°C. So far we have not detected this resistant mutation in field strains. This procedure has correctly identified the resistance status of all strains for which we have bioassay and sequence data.

Discussion

Single base-pair mutations in *R. secalis* causing amino acid substitutions and correlated with benzimidazole resistance, were similar to those found in resistant field strains of other plant pathogenic fungi (Koenraadt & Jones, 1992; Martin, et al, 1992; Yarden & Katan, 1993). In both *R. secalis*, *Botrytis cinerea* (Yarden & Katan, 1993), and *Venturia inaequalis* (Koenraadt, *et al*, 1992) substitution of a lysine for glutamic acid results in a loss of the negatively correlated cross-resistance between benzimidazoles and diethofencarb, although this mutation has yet to be found in field populations of *Rhynchosporium*.

ASO probes provide a rapid diagnostic technique which takes less than 24h to complete. At present, the method requires DNA template extracted from pure cultures of *R. secalis*, but we are currently exploring nested primer PCR approaches for direct

detection from diseased leaves. This should reduce the time taken to obtain a result from field samples to 24-48h, compared with 6-8 weeks now required for conventional isolation and bioassay. This technology not only provides an accurate detection of resistance but can distinguish between different resistant alleles. Although up to 10 sites within the β-tubulin gene have been identified as conferring benzimidazole resistance (Osmani & Oakley, 1991), it is clear that mutations in field strains of phytopathogens are restricted to codons 198 and 200 (Koenraadt & Jones, 1992; Adachi, *et al*, 1993; Yarden & Katan, 1993). Consequently, the number of different ASO probes needed to monitor field populations is quite limited; only four probes were needed to accurately identify benzimidazole resistance in a large world-wide collection of *V. inaequalis* strains (Koenraadt & Jones, 1992). For obligate or slow growing pathogens rapid detection of resistance using ASO probes should allow more samples to be tested and so contribute to more effective monitoring of the performance of strategies used to combat resistance. Where efforts to assess the risk of resistance to new compounds identifies resistant mutants at an early stage in development, rapid, DNA based, detection offers a more effective way to identify rare mutants in field populations, than conventional monitoring methods.

Acknowledgements

We thank MAFF and H-GCA for funding this work.

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FIGURE 1. Allele-specific oligonucleotide DNA probes

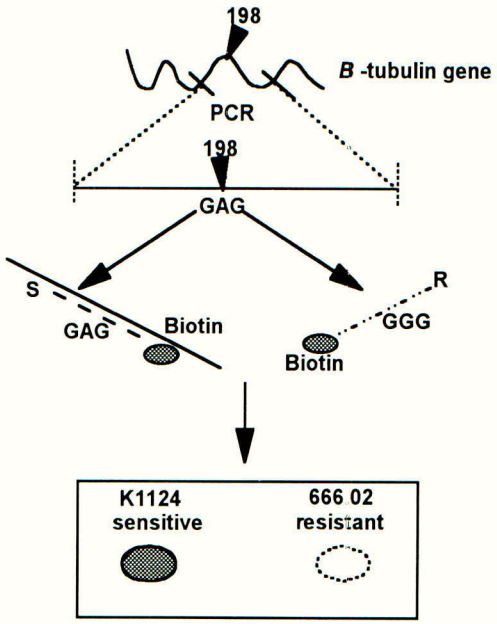

TABLE 1. DNA changes at codon 198 in the *B*-tubulin, and benzimidazole resistance in *Rhynchosporium secalis*..

Isolate	Amino acids						Fungicide sensitivity	
	196 SER	197 ASP	198 GLU	199 THR	200 PHE	201 CYS	Benzimidazoles	N-phenyl-carbamate
K1124	TCT	GAT	*GAG*	ACC	TTC	TGT	*S*	*R*
809.02	TCT	GAT	*GAG*	ACC	TTC	TGT	*S*	*R*
666.02	TCT	GAT	GLY *GGG*	ACC	TTC	TGT	*R*	*S*
769.03.03	TCT	GAT	*GGG*	ACC	TTC	TGT	*R*	*S*
BEN22	TCT	GAT	LYS *AAG*	ACC	TTC	TGT	*R*	*R*

MUTATIONS IN THE BETA-TUBULIN GENE OF BENOMYL-RESISTANT PHENOTYPES OF *BOTRYTIS CINEREA*

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Three phenotypes were identified among benomyl-resistant strains of *Botrytis cinerea* in Israel, when tested for sensitivity to carbendazim (MBC) and diethofencarb (NPC): $Ben^{HR}NPC^{S}$= highly resistant to MBC (EC_{50}>50 µg/ml) and sensitive to 0.5µg/ml NPC; $Ben^{MR}NPC^{R}$= moderately resistant to MBC ($10 \leq EC_{50} < 20$ µg/ml) and resistant to 10 µg/ml NPC; and $Ben^{HR}NPC^{R}$= highly resistant to MBC and resistant to NPC. A 1-kb fragment of the wild-type gene encoding for beta-tubulin (designated *benA*) in *B. cinerea* was cloned and sequenced. The deduced partial amino acid sequence of the *B. cinerea* beta-tubulin showed a high degree of similarity to beta-tubulins of other filamentous fungi. A PCR approach was used to amplify and sequence 992-bp *benA* fragments from strains representing the three phenotypes. In the eight Ben^{R} strains analyzed, three single base-pair mutations were identified and found to correlate with the different phenotypes: codon 198, encoding glutamic acid in the wild type, was changed to an alanine codon in the $Ben^{HR}NPC^{S}$ phenotype, or to a lysine codon in the $Ben^{HR}NPC^{R}$ phenotype; codon 200, encoding phenylalanine was changed to a tyrosine codon in the $Ben^{MR}NPC^{R}$ phenotype. These mutations were similar to those identified in benomyl-resistant field strains of other phytopathogenic fungi.

RESISTANCE OF *BOTRYTIS CINEREA* TO DICARBOXIMIDES, BENZIMIDAZOLES AND PHENYLCARBAMATES IN THE CHAMPAGNE VINEYARDS

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ABSTRACT

Strains of *B. cinerea* resistant either to dicarboximides (e.g. iprodione, procymidone, vinclozolin) or to both benzimidazoles (e.g. carbendazim) and phenylcarbamates (e.g. diethofencarb) are commonly found in Champagne vineyards. Limitations in the use of these fungicides are recognized as anti-resistance strategies. However, when the frequencies of resistant strains are too high, they can be temporarily withdrawn. Such advice is given according to the results of the monitoring done every year at vintage.

INTRODUCTION

In french vineyards, *B. cinerea* remains a parasite feared by vine-growers because of its qualitative and quantitative effects on wine production. The chemical control of this fungus is normaly achieved by three of four treatments applied between the flowering stage and three weeks before vintage. Several families of fungicides are registered in France. The oldest ones are protectants (e.g. chorothalonil, dichlofluanid, folpet, thiram) and have never been affected by resistance because of their multi-site effects. On the other hand, resistance developed towards dicarboximides (e.g. clozolinate, iprodione, procymidone, vinclozolin), benzimidazoles (e.g. benomyl, carbendazim, thiophanate-methyl) and phenylcarbamates (e.g. diethofencarb). The evolution of such phenomena in Champagne vineyards over 10 years and the anti-resistance strategies will be presented in this paper.

CHARACTERISTICS OF THE VARIOUS RESISTANT STRAINS

Dicarboximides are among the most effective fungicides against *B. cinerea*. However the development of moderately resistant strains (Rd ; Table 1) provided inadequate control of grey mould in Champagne vineyards and also in many other French regions (Leroux and Clerjeau, 1985).

Benzimidazoles which were introduced before dicarboximides, selected highly-resistant strains which were very susceptible to the phenylcarbamate diethofencarb (Rb1 ; Table 1). Since the commercialization of mixtures diethofencarb/carbendazim in 1987, a new phenotype simultaneously-resistant to these two fungicides appeared (Rb2 : Table 1).

All strains remained sensitive to pyrimethanil, an anilinopyrimidine recently introduced in France (Table 1).

TABLE 1 : Effects of fungicides on the germ-tube elongation of various strains of B. cinerea[a] collected in Champagne vineyards in 1992 and 1993

Fungicides	Mean EC50 (mg/l) of phenotypes				
	Sd	Rd	Sb	Rb1	Rb2
iprodione	0.8	5.0	-	-	-
procymidone	0.6	8.0	-	-	-
vinclozolin	0.4	5.0	-	-	-
carbendazim	-	-	0.05	>25	5.0
diethofencarb	-	-	>25	0.05	>25
pyrimethanil	0.07	0.07	0.06	0.07	0.07

[a] : the tests were conducted according to the method of Leroux and Gredt (1989) on four to eight strains of each type.

TABLE 2 : Relationship between the percentages of B. cinerea resistant strains and the efficacies of a programme combining several fungicides (average values obtained in 3 or 4 trials each year)

years	Control plots		Treated plots[a]
	% Rd	% Rb2	% efficacy
1989	25	1	50
1990	31	14	72
1991	38	34	13
1992	20	33	17

[a] : the programme consisted in three applications of a mixture carbendazim/diethofencarb (500 + 500 g/ha) followed by a mixture vinclozolin/thiram (500 + 3200 g/ha) and a dicarboximide alone (procymidone or vinclozolin at 750 g/ha).

RESISTANCE PHENOMENA IN FIELD TRIALS

From the various trials conducted in Champagne region over the last six years, it has been shown that :

- the selection pressure induced by one application of procymidone or vinclozolin (750 g/ha) towards Rd strains was lower than that of a mixture of carbendazim/diethofencarb (500 + 500 g/ha) towards Rb2 strains (Leroux and Moncomble, 1993).

- at reduced rates (500 g/ha instead of 750 g/ha) procymidone and vinclozolin used in mixture with thiram (3200 g/ha) exerted a selection pressure significantly lower than that of dicarboximides applied alone at their full rate (750 g/ha) (Leroux and Moncomble, 1993).

- the programme based on three applications of a mixture carbendazim/diethofencarb, followed by a mixture vinchlozolin/thiram, and a dicarboximide alone was effective in 1989 and 1990 but failed to control grey mould in 1991 and 1992. It seemed that this was mainly due to the development of Rb2 strains (Table 2).

MONITORING AND RECOMMENDATIONS OF FUNGICIDE USE

A monitoring of resistance of B. cinerea is conducted every year in more than 100 commercial Champagne vineyards at vintage. In each location, 10 to 20 diseased berries are collected and the percentages of the various phenotypes are determined according to the method of Leroux and Clerjeau (1985).

TABLE 3 : Evolution of number of annual fungicide treatments and percentages of B. cinerea resistant strains in Champagne vineyards between 1982 and 1993

years	dicarboximides		carbendazim and diethofencarb		
	Tre.[a]	% Rd	Tre.[a]	% Rb1	% Rb2
1982	4	87	0	85	0
1983	0	72	0	82	0
1984	0	42	0	76	0
1985	0	22	0	94	0
1986	0.5	21	0	97	0
1987	0.8	30	0.8	97	0
1988	0.8	22	1.5	95	2
1989	1.1	30	1.1	77	21
1990	1.6	37	0.9	52	46
1991	1.7	48	0.9	38	62
1992	1.5	56	0.2	54	46
1993	0	34	0	70	29

[a] : average number of annual treatments with dicarboximides (alone or in mixture with thiram) or mixtures carbendazim/ diethofencarb.

From the results of Table 3, it appeared that the frequencies of dicarboximide-resistant strains (Rd) as well as those of strains doubly resistant to carbendazim and diethofencarb (Rb2) decreased when the selection pressure ceased (this was not the case with the Rb1 strains). This non- persistant resistance which is probably due to reduced fitness of Rd and Rb2 strains permitted a discontinious use of both dicarboximides and mixtures carbendazim/diethofencarb. The re-employment of these fungicides occured when the average percentages of resistant strains were below 25 % at the preceeding vintage.

This survey confirmed than one annual application of dicarboximides (alone or in mixture with thiram) exerted lower selection pressure than that of mixtures carbendazim/diethofencarb (Table 3).

CONCLUSION

In spite of the limitation of annual treatments against B. cinerea (three instead of four or five in the 1980's) and of combined uses of the varicus available fungicides (mixtures or alternations), the chemical control of grey mould remains uncertain in Champagne vineyards. As an example, the recommendation for 1994, advises the successive applications of a mixture carbendazim/diethofencarb, of pyrimithanil and of a dicarboximide (alone or in mixture with thiram). This may lead to selection of RB2 strains and possibly Rd ones, which is likely to lead (once more) to the withdrawal of mixtures carbendazim/diethofencarb and dicarboximides in 1995. Other considerations, such as residue levels or negative effects on fermentation are additional constraints for the establishing of grey mould treatment programmes.

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DISTRIBUTION AND INCIDENCE OF BENZIMIDAZOLE-RESISTANT FUSARIUM SAMBUCINUM AND HELMINTHOSPORIUM SOLANI ISOLATED FROM POTATO IN NORTH AMERICA.

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ABSTRACT

Isolates of Fusarium sambucinum and Helminthosporium solani were recovered from dry rot and silver scurf diseased potatoes collected from states and provinces throughout the United States, Mexico, and Canada. Single spore cultures of both fungi were prepared and tested for resistance to thiabendazole (TBZ) by growth reduction (ED50) on TBZ-amended acid PDA (Fusarium) or modified V-8 (H. solani) media. Of 504 Fusarium isolates representing 18 states, 378 or 75% of them, were resistant to TBZ. The resistance ranged from 1 to 64 ppm. The Fusarium isolated was almost exclusively F. sambucinum. Of the H. solani isolates tested representing 7 states, 21/27 or 77% were resistant to TBZ. The resistance ranged from 20 to 50 mg/l. TBZ-resistant isolates were cross-resistant to thiophanate-methyl.

INTRODUCTION

Storage diseases of potato can cause serious problems costing producers and processors large amounts of time and money. Many of these diseases can be controlled by post-harvest application of fungicides as the potatoes are going into storage. The only post harvest chemical approved for potatoes in the US is thiabendazole ('Mertect 340F'). This chemical has been intensively used and has provided excellent primary control of dry rot, caused by Fusarium sambucinum (Fs), for 20 years, and assumed control of silver scurf, caused by Helminthosporium solani (Hs), even though it is not a target disease for this fungicide. Recent outbreaks of both dry rot and silver scurf prompted us to re-examine these diseases, and the role that fungicide application has on disease management, including possible fungicide resistance to TBZ.

METHODS AND MATERIALS

Isolations

Fusarium was isolated from dry rot diseased potato samples of various cultivars collected from throughout the US, Mexico, and Canada. Most samples were sent by colleagues at universities and industry. Fusarium was successfully isolated from samples representing 18 states or provinces (Table 1). Potatoes were surface sterilized (0.5% Na hypochlorite, 15 min) and small pieces of tuber tissue at the margin of the dry rot lesion placed on acidified potato dextrose agar (APDA). After 5-7 days incubation, Fusarium cultures were transferred to PDA medium. Single spore cultures of each isolate were

prepared. 504 Fs cultures were collected for testing (Table 1). Carnation leaf agar was used to identify isolates (Nelson et al, 1983).

Hs was isolated from naturally-infected silver scurf tubers recovered from samples collected or sent from 7 states or provinces (Table 2). Surface sterilized tubers, (0.5% Na hypochlorite, 15 min), were incubated in a humid chamber at room temperature. After 5-7 days, conidia were picked from the tuber surface and spread on APDA. After 24-58 hrs, germinated single spores were transferred to modified V-8 agar medium.

Resistance testing

Single spore Fs colonies were tested for TBZ resistance using PDA amended with technical grade TBZ (98.5%), provided by Merck & Co., Rahway, NJ, at 0, 10, 20, 50 and 100 mg/l. Mycelial disks 5 mm in diameter were removed from Fs cultures 5-7 days old, and a single disk was centered in petri plates with or without TBZ. Cultures were incubated at room temperature (approx 25°C) for 7 days under continuous fluorescent light. Each treatment was replicated three times; the experiment was repeated twice.

Single spore Hs colonies were tested for TBZ resistance using modified V-8 (MV8) medium amended by the addition of technical grade TBZ as above. TBZ concentrations ranged from 0 to 200 ppm. Single mycelial disks 5 mm in diameter were removed from the edge of one-month-old cultures and centered in petri dishes with or without TBZ. Cultures were incubated 21 days at 23°C in the dark. Each treatment was replicated four times; the experiment was repeated twice.

Calculation of TBZ resistance

Resistance data was based on the inhibition of radial mycelial growth of the colony after 7 (Fs) or 21 (Hs) days. Two diameter measurements were taken for each colony at perpendicular axes to each other and averaged. The logarithm of the concentration versus the logarithm of the percentage growth reduction was plotted and regression analysis used to determine graphically the concentration in mg/l of the TBZ causing 50% reduction of colony growth compared to the unamended control; this was defined as ED50 (Trivellas, 1988).

RESULTS

Of the Fs isolates recoverd from potato tubers with dry rot, greater than 95% were identified as Fusarium sambucinum. The results of TBZ resistance testing of these isolates can be seen in Table 1. Overall, 378/504 isolates tested, or 75%, were resistant to TBZ. The ED50 ranged from 1-64 mg/l.

The results of resistance testing with Hs isolates can be seen in Table 2. Of the isolates of Hs recovered from silver scurf affected tubers, 21/27, or 77%, were resistant to TBZ. Resistance ranged from 20-50 mg/l.

CONCLUSION

It is evident that resistance to TBZ is geographically widespread in fungi causing the post- harvest diseases dry rot and silver scurf in North America. This data should be used in formulating management strategies for control of these diseases.

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TABLE 1. Distribution and frequency of TBZ-resistant Fusarium[1] isolates in North America

Isolate Source	Number Tested	Percent Sensitive	Percent Resistant	ED50[2]
North Dakota	76	18	82	30
Minnesota	30	30	70	21
South Dakota	2	100	0	< 10
Idaho	255	16	84	27
Wisconsin	9	78	22	25
Maine	4	100	0	< 10
Texas	1	100	0	< 10
Washington	7	100	0	< 10
Alaska	4	75	25	53
Colorado	15	93	7	21
New York	39	3	97	29
Michigan	17	0	100	33
Nebraska	12	25	75	32
Alabama	4	100	0	< 10
Utah	5	0	100	31
Delaware	2	100	0	< 10
Wyoming	7	29	71	29
Pr. Ed. Is., Canada	4	100	0	< 10
Michoacan, Mexico	12	66	33	13
Total	504	25	75	

[1]Almost exclusively F. sambucinum

[2]Estimated using colony diameter reduction after seven days

TABLE 2. Frequency and distribution of TBZ-resistant Helminthosporium solani isolates from North America

Isolate Source	Number Tested	Percent Sensitive	Percent Resistant	ED50[1]
North Dakota	14	7	93	20-47
Minnesota	2	50	50	50
Wisconsin	2	100	0	--
Alaska	1	100	0	--
Oregon	1	100	0	--
New Brunswick	5	0	100	25-32
Maine	2	0	100	20-32
Total	27	22	78	

[1]Estimated using colony diameter reduction after 21 days

IMPACT OF BENZIMIDAZOLE-RESISTANT FUSARIUM SAMBUCINUM ON DRY ROT DISEASE OF STORED POTATOES.

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ABSTRACT

Isolates of Fusarium sambucinum (Fs) with high, medium, and low resistance to thiabendazole (TBZ) or susceptible were evaluated for their impact on dry rot incidence and severity of inoculated potatoes. Potato tubers, cv. Norchip, were mechanically bruised, inoculated with Fs spores, treated with TBZ by dip or low-volume spray, and evaluated after 2,3,4 and 5 months' storage at 10°C. The experiment was conducted twice. In most instances, TBZ resistance resulted in increased incidence and severity of dry rot compared to the control treatments. Method of TBZ application did not appreciably affect the incidence or severity of dry rot in stored potato tubers.

INTRODUCTION

Previous work (Secor et al., these proceedings) has shown widespread resistance of Fusarium sambucinum (Fs) to the fungicide thiabendazole (TBZ). Of 504 isolates of Fs tested, 378, or 75% of them were resistant. The ED50 ranged from 1 to 64 mg/l. TBZ is the only fungicide registered for post-harvest application and has specific activity against Fusarium on a number of crops. Dry rot disease has recently become a serious problem of stored potatoes after twenty years of excellent control which coincides with the widespread use of TBZ. Because fungicide resistance is usually irreversible, it was of interest to determine if the resistance observed has an impact on the ability of TBZ to control dry rot. Similar studies (Hall and Hide, 1992) have shown that resistance to TBZ in Helminthosporium solani does reduce the ability of post-harvest application of TBZ to control silver scurf. The purpose of these studies was to determine the role TBZ resistance in Fs may play in the development of dry rot disease in stored potatoes.

METHODS AND MATERIALS

Potato tubers, cv. Norchip, were used in all studies. In the first year of the study, potato tubers were inoculated with one of eight isolates of Fs that varied in their response to TBZ and could be categorized as susceptible (<10 mg/l) or resistant to low (15-20 mg/l), medium (25-30 mg/l) or high (>40 mg/l) levels of the fungicide. Four isolates of each TBZ response category were recovered from dry rot infected tubers originating from North Dakota and four were from Idaho.

Potato tubers were bruised in a small batch cement mixer for 60 seconds prior to inoculation. Fusarium cultures used for inoculum were grown on PDA at room temperature under continuous fluorescent light. Inoculum was prepared from 5-7 day old single spore

cultures of each isolate and diluted to 300,000 propagules/ml. Following inoculation with Fs, the potatoes were treated in one of several ways. The treatments were: untreated (no TBZ), water dip (20 sec), TBZ dip (label rate), TBZ at ultra-low volume spray (label rate). Controls used to monitor natural, background levels of dry rot infection included a) unwashed, unbruised, uninoculated, untreated tubers; b) washed unbruised, uninoculated, untreated tubers; c) unwashed, bruised, uninoculated, untreated tubers and; d) washed, bruised, uninoculated, untreated tubers.

After inoculation and treatment in the above manner, tubers were allowed to air dry. One hundred tubers of each treatment combination (isolate X chemical treatment) were placed into four mesh bags (25 tubers/bag) and placed into a humidified storage (>90% RH) at 10°C. Tubers from each treatment were evaluated for incidence and severity of dry rot after 2, 3, 4 and 5 months of storage. At each sampling date, five tubers/treatment were frozen (-30°C) and sent to Merck Co. (Rahway, NJ) for TBZ residue analysis. Additional tubers representing each isolate were also randomly selected among the treatments at each sampling date. Tuber tissue from the margins of decay was used to isolate the causal Fs. Fusarium isolates obtained in this manner were screened for TBZ resistance and used to make comparisons with isolates originally used in inoculations.

The experiment was repeated with the exception that the two Fs isolates with high levels of TBZ resistance were compared to two isolates of Fs susceptible to TBZ. Dry rot incidence and severity were evaluated 2 and 3 months after inoculation.

RESULTS AND DISCUSSION

Background levels of naturally-occurring Fusarium were insignificantly low at each sampling date on the potato tubers used in the study. Therefore, these treatments were deleted in subsequent analyses. Furthermore, random isolations and TBZ screening demonstrated that the dry rot observed during the course of the study was caused by the isolates used during inoculations.

In the first trial, the incidence of dry rot lesions in potato tubers inoculated with Fs isolates resistant to TBZ was significantly higher than in tubers inoculated with TBZ-susceptible isolates (Figure 1A). The severity of dry rot, as determined by the percentage of tuber tissue rotted, was also greater in tubers inoculated with TBZ-resistant Fs isolates (Figure 1B). However, these differences were only statistically significant at the first and last sampling date.

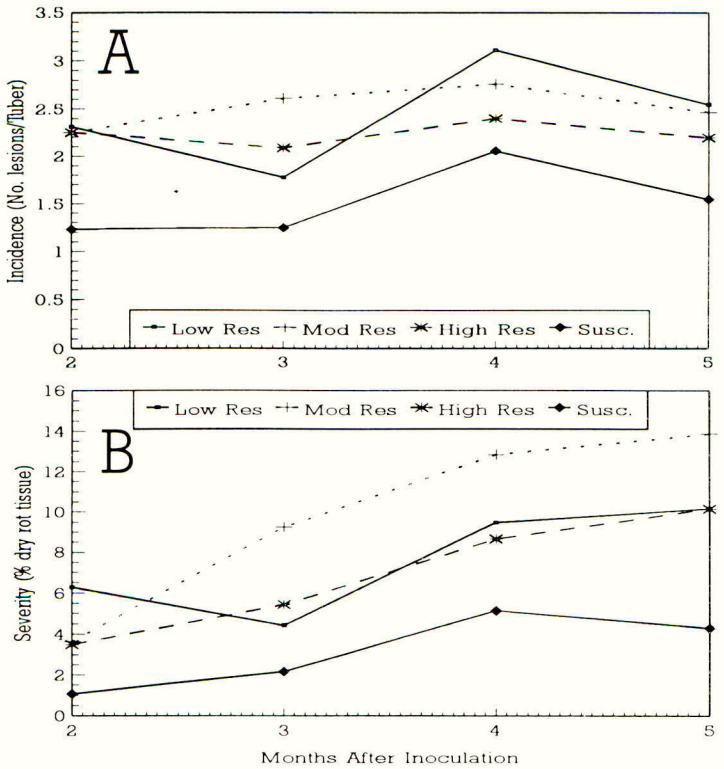
Similar trends were observed in the second trial when only four isolates were used; however, differences were significant only on the second sampling date. These results indicate that the ability of a Fs isolate to resist TBZ may give it a selective advantage in a mixed population of isolates. This could occur if the fungicide is being used on a farm. Differences in isolate aggressiveness, however, may be responsible for increased severity of disease in addition to fungicide resistance. There is, however, no change in mating type, toxin production, vegetative compatibility or ability to grow *in vitro* as a result of TBZ resistance (Desjardins, et al. 1993).

Few differences were detected in the development of dry rot due to method of TBZ application in the first trial (Figure 2). The incidence and severity of dry rot was not significantly different between tubers treated with TBZ by dipping or by ultra-low volume spray, regardless of sampling date. These results were obtained despite the fact that residue on the periderm of tubers dipped in TBZ (38-42 mg/l) was three times higher than tubers sprayed with TBZ at ultra-low volumes (11-14 mg/l). These results were duplicated in the second trial. This was interpreted to mean that the recent increase of dry rot in stored potatoes is not due to poor coverage of TBZ fungicide applied at ultra-low volumes.

It was observed that the incidence and severity of dry rot in untreated potato tubers was generally not significantly different from TBZ-treated tubers in the first trial (Figure 2). This was unexpected and no explanation can be given for these results. However, in the second trial, the incidence and severity of dry rot in untreated tubers was always significantly higher than in TBZ-treated potato tubers.

It is obvious from these studies that application of TBZ can no longer be relied upon exclusively for the control of Fusarium dry rot in stored potatoes. An integrated approach including wound reduction of potato tubers during harvest and storage conditions that promote wounding healing must be used to effectively manage this disease.

Figure 1. Incidence and severity of dry rot caused by isolates of Fusarium sambucinum differing in response to TBZ.



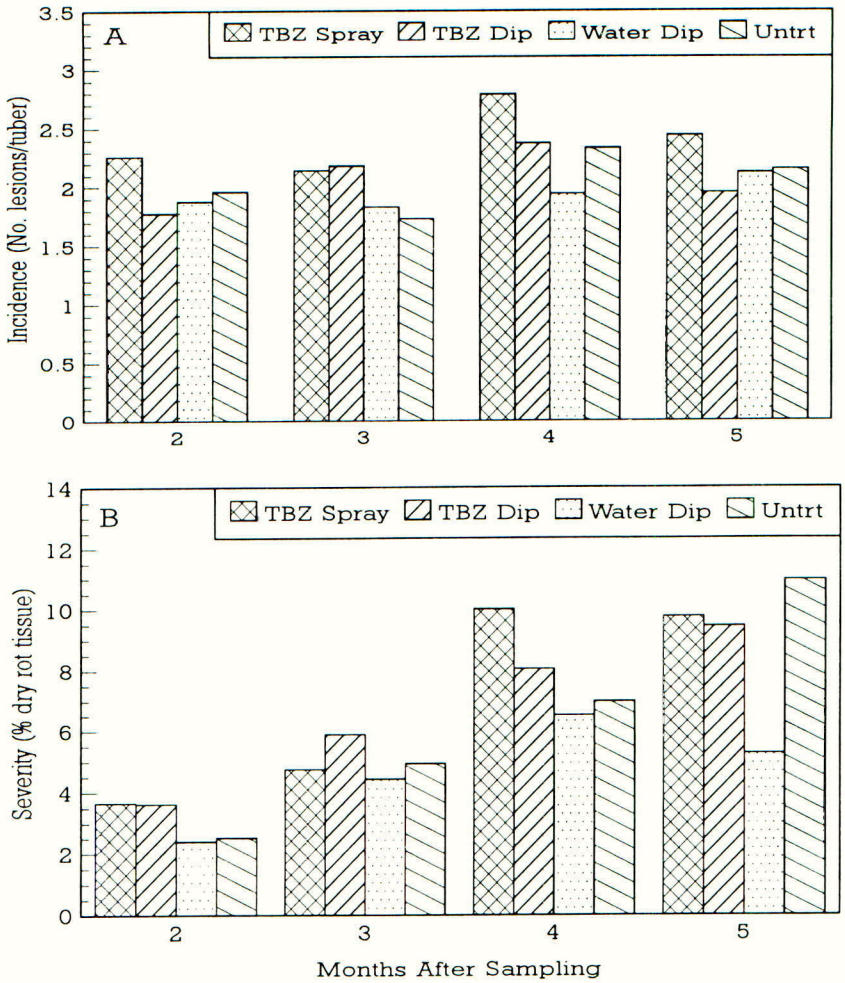


Figure 2. **Effect of post-inoculation treatment; untreated, water dip or TBZ applied by ultra-low volume spray or by dipping, on the incidence and severity of dry rot at four sampling dates.**

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