REDUCED SENSITIVITY TO CARBENDAZIM IN ISOLATES OF *BOTRYTIS ALLII*

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ABSTRACT

Fifty isolates of *Botrytis allii*, causing onion botrytis neck rot, were obtained from onion seed and bulbs throughout New Zealand and tested for their sensitivity to carbendazim (benzimidazole) fungicide in a mycelial growth study. Agar plates amended with carbendazim at 6 or 8 different concentrations were inoculated with an 8 mm disc of mycelium of each of the isolates and incubated in the dark at 20°C. Radial growth of the colonies was measured after 4 days. EC_{50} (effective concentration required to give half of maximum growth) values were estimated by fitting logistic curves, with growth as a proportion of the maximum growth. Of the 50 isolates, 16 were identified as resistant, 32 as sensitive and two had a variable response. The estimated EC_{50} values for the isolates that were sensitive ranged from 0.006 to 0.053 µg/ml active ingredient. It is recommended that New Zealand onion growers closely follow the current benzimidazole resistance management strategy.

Keywords: botrytis neck rot, *Botrytis allii*, carbendazim, benzimidazole, fungicide resistance, fungicide sensitivity.

INTRODUCTION

Botrytis neck rot, caused by the fungus *Botrytis allii* Munn. (synonym *B. aclada* Fresenius subgroup AII, Yohalem et al. 2003), is a troublesome disease of onion due to its latent nature in the field, becoming evident only after 3–4 months of storage (Maude & Presly 1977a,b), often after shipments reach an overseas port. The first sign of neck rot is a watery decay in the neck of the onion, which moves downwards causing scales to become soft, watery and translucent. The necks of affected bulbs soften and become sunken, a white to grey mycelial growth eventually develops between the bulb scales and masses of black sclerotia may develop on the outer scales around the neck. Neck rot is often exacerbated by secondary pathogens that occur in storage.

Infection by *B. allii* can occur at any stage of the onion life cycle. Infected seed has been indicated as the main source of infection of onion bulbs in storage as levels of seed infection can correlate with subsequent levels of bulb disease (Maude & Presly 1977a,b; Stewart & Franicevic 1994). The fungus invades seedling cotyledons and remains symptomless in the leaf tissue until it colonises the tissue at the necks of bulbs, causing neck rot. Other potential sources of infection in onion crops are air-borne conidia originating from volunteer onions, infected debris and cull-piles or sclerotia (resting bodies) surviving in plant debris and soil (Maude 1983). Disease progress is also affected by weather conditions during the season. Neck rot can be particularly severe if prolonged wet periods occur during curing when onion necks are still succulent. Wounds also provide entry points for the pathogen.

The control of onion neck rot has largely comprised seed treatment with carbendazim (chemical group benzimidazole) and spray applications to onion crops of thiophanate-methyl (chemical group benzimidazole), mancozeb (chemical group dithiocarbamate), triadimenol (chemical group trizole) as well as carbendazim. The New Zealand onion industry has reported the increased incidence of neck rot in onion bulbs in recent years as well as suspected occurrences of resistance to carbendazim fungicide. Resistance in

Botrytis allii to carbendazim has been reported in Australia (Metcalf & Hills 2004). The objective of the work reported here was to test isolates of *Botrytis allii* for resistance to carbendazim fungicide in a laboratory-based mycelial growth Petri plate assay.

MATERIALS AND METHODS

Fifty *B. allii* isolates were obtained from infected seed (22 isolates) and onion bulbs (28 isolates) sent by seed companies, exporters and growers during the 2005–06 growing season mainly from Canterbury, Blenheim and Pukekohe (isolates 34-45 were isolated from seed that originated in Australia) as part of the MAF Sustainable Farming Fund project on Allium crops (Marroni et al. 2006). The isolates were kept on potato-dextrose agar (PDA, Difco Laboratories, Sparks MD, USA) slants at 5°C in the Crop & Food Research plant pathology mycology collection until testing for carbendazim resistance. A commercial formulation of carbendazim (Prolific® 500 g/litre carbendazim and 5 g/litre formaldehyde) was used. The isolates were transferred from slants and grown on PDA Petri plates at 20°C in the dark. Using a cork borer, agar discs of 8 mm were obtained from the active growing border of 7-day-old cultures from each of the 50 isolates and placed upside down in the centre of Petri plates containing PDA with either no fungicide or amended with the fungicide carbendazim. There were three replicates and each replicate consisted of one Petri plate for each isolate and concentration. There were six carbendazim concentrations in the first replicate (0, 0.01, 0.1, 1, 10 and 100 µg/ml active ingredient (ai)). In the second and third replicate, isolates that showed resistance to carbendazim in the first replicate were also tested at concentrations of 250 and 500 µg/ml ai. Petri plates were incubated at 20°C in the dark for 4 days.

Radial growth was determined by measuring the diameter of growth across the centre of the disc on two transversal positions to obtain a mean diameter. Diameter of the original disc (0.8 mm) was subtracted from the mean diameter growth to obtain the actual colony growth. The effective concentration required to give half of maximum growth (EC_{50}) values were estimated by fitting logistic curves, with diameter as a proportion of the maximum diameter. This was done using generalised non-linear modelling procedures, as implemented in GenStat's PROBIT analysis procedure (Genstat Committee 2006), using a logistic of ln(concentration) to describe the concentration response. This curve has three parameters: EC_{50}, b (relating to the steepness of the curve) and maximum growth. For the isolates where growth reached the edge of the plate, the maximum growth did not need to be estimated; it was fixed to the edge of the plate. For isolates for which growth did not reach the edge of the plate, maximum growth was estimated (there were no isolates for which growth at 0 was not the maximum). In all analyses, it was assumed that for a high enough concentration, there would be no growth of the isolate. At the highest dose of carbendazim, growth for the resistant isolates was little changed from the growth at 0 carbendazim, so EC_{50} could not be estimated for these isolates. There were data for only one replicate each for isolates 13, 47 and 48. There were data for two replicates for isolates 28, 43, 46, 53, 55, 57, 58, 59, 61 and 62. In some cases, the missing data occurred because of contamination, or because the isolate did not grow on that plate. The isolate 51 was resistant in replicates 2 and 3, but sensitive in replicate 1.

RESULTS

From the 50 isolates tested, 32 were identified as sensitive, 16 as resistant and two isolates had a variable response to carbendazim (Table 1). The estimated EC_{50} for all sensitive isolates ranged from 0.006 to 0.053 µg/ml and followed a similar growth pattern as shown for sensitive isolates 2 and 23 (Fig. 1). No growth was observed for sensitive isolates above 0.1 µg/ml carbendazim. Therefore isolates were considered sensitive when colony diameter at 0.1 µg/ml was less than 50% of the unamended medium. Radial growth of the 16 resistant ($EC_{50} > 1.5$ µg/ml) isolates was unaffected by the carbendazim concentrations tested as demonstrated for isolate 40 (Fig. 1). This stark contrast in growth for sensitive and resistant isolates is also depicted in Figure 2. In other studies, isolates were considered resistant if they were able to grow in agar plates containing

1.0 µg/ml (Metcalf & Hills 2004) or 2.0 µg/ml (Gladders et al. 1994) of benzimidazole fungicide. Only two (40 and 41) out of 22 isolates from seed were identified as resistant, both of these coming from the same seed line. The remaining resistant isolates were obtained from onion bulbs (Table 1). Isolate 47 had a variable response with an estimated EC_{50} of 1.03 µg/ml carbendazim (Fig. 1) based on one replicate. Isolate 51 also had a variable response and showed resistance to carbendazim in two replicates and sensitivity in one replicate.

TABLE 1: Estimated EC_{50} (µg/ml active ingredient) for sensitive, resistant and variable response isolates of *Botrytis allii* from seed or bulb used for testing sensitivity to carbendazim fungicide. Standard errors are in parenthesis, degrees of freedom varied from 9 to 41.

	Isolate from seed		Isolate from bulb	
Isolate resistance	No.	EC_{50}	No.	EC_{50}
Sensitive < 0.1 µg/ml	2	0.024 (0.019)	1	0.046 (0.003)
(n=32)	3	0.019 (0.001)[1]	21	0.029 (0.003)
	4	0.040 (0.007)	22	0.045 (0.013)
	5	0.025 (0.001)[1]	23	0.040 (0.005)
	6	0.013 (0.014)[1]	24	0.037 (0.003)
	8	0.028 (0.004)	26	0.020 (0.001)[1]
	9	0.032 (0.004)	27	0.036 (0.004)
	30	0.017 (0.003)	28	0.030 (0.004)
	34	0.026 (0.007)	55	0.030 (0.005)
	35	0.053 (0.013)	56	0.034 (0.003)
	36	0.032 (0.007)	57	0.048 (0.018)
	37	0.043 (0.013)	58	0.016 (0.005)
	38	0.015 (0.003)	59	0.0059
	39	0.019 (0.001)		
	42	0.029 (0.003)		
	43	0.046 (0.002)		
	44	0.033 (0.008)		
	45	0.046 (0.017)		
	46	0.018 (0.002)		
Resistant >1.5 µg/ml (n=16) – isolates had no reduction in growth at any carbendazim rate	40, 41		13, 14, 15, 16, 17, 19, 25, 31, 33, 48, 49, 53, 61,62	
Variable response (n=2)	51[2]		47	1.03 (1.50)

[1]Indicates estimates where isolate growth at 0 concentration was less than growth for some concentrations of fungicide, and where growth at zero was substituted with growth to the edge of the dish.
[2]Indicates an isolate that was resistant in replicates 2 and 3, sensitive in replicate 1.

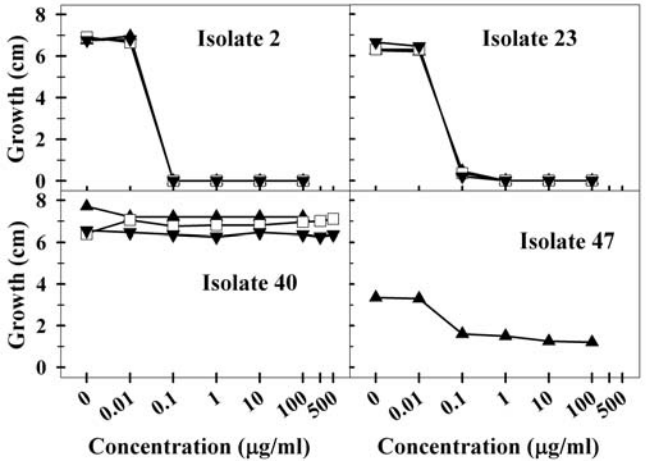

FIGURE 1: Radial growth (cm) at different carbendazim concentrations for sensitive (isolates 2 and 23), a resistant (isolate 40) and a moderately resistant (isolate 47) isolates of *Botrytis allii*. The replicate experiments for each isolate are shown as separate lines.

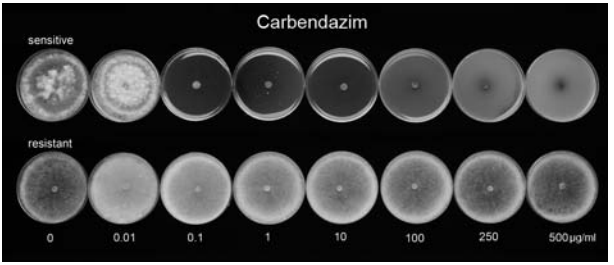

FIGURE 2: Photographs showing radial growth of a sensitive (isolate 34) and resistant isolate (isolate 40) of *Botrytis allii* on carbendazim-amended agar plates after 4 days incubation in the dark at 20ºC.

DISCUSSION

This study is the first report in the literature confirming that isolates of *Botrytis allii* have resistance to the fungicide carbendazim in New Zealand.

Benzimidazole fungicides include benomyl, carbendazim, fuberidazole, thiabendazole, thiophanate and thiophanate methyl. They were introduced in the 1960s and 1970s and were among the first broad-spectrum plant disease control agents, providing excellent preventive, curative and systemic activities (Hewitt 1998). However, their mode of action comprised a single site, involving inhibition of fungal tubulin polymerisation, and their widespread and extensive use led to the development of resistance in many crops. This was the first group of selective fungicides for which resistance in plant pathogens was found and they are no longer effective for many important diseases (Hewitt 1998). Resistance to benzimidazoles is very persistent and is controlled by a single major

gene. However, the benzimidazoles are still important and useful in some situations, particularly for one-off applications where there is no need for continued use of the fungicide (Beresford 2005).

Resistance to benzimidazole fungicides (benomyl and carbendazim) in *B. allii* has been reported in onions from Israel since 1983 (Kritzman 1983), in shallots in the UK (Gladders et al. 1994) and in onions in Australia (Metcalf & Hills 2004). In the UK, benzimidazoles are no longer recommended for the control of neck rot on shallots (Anon. undated).

Worldwide, carbendazim is still regarded as a highly effective seed treatment. During this study, low levels of *B. allii* were detected in seed and only two isolates out of 22 were classed as resistant. Both of these isolates came from the same seedline, which originated in Australia. However, only nine seed lines were tested in this study and a more extensive collection of *B. allii* isolates from seed lines has commenced.

Most resistant isolates were obtained from bulb samples, which may be the cause of loss of effectiveness of treatments in the field, leading to higher levels of neck rot on stored onions. Increased control failures in New Zealand onion crops may be exacerbated by the use of carbendazim in both seed and the subsequent bulb production. Development of resistance to benzimidazole fungicides is linked to routine applications over time (Hewitt 1998). Carbendazim label recommendations state that no more than 2 applications should be used per season for onion botrytis control to avoid development of resistance but it is not uncommon for growers to apply up to 6 applications of benzimidazole fungicides to a bulb crop in one season (S.L.H. Viljanen-Rollinson, unpubl. data). The current benzimidazole resistance management strategy (Beresford 2005) states:

- where resistant strains of the pathogen are known, or are likely to occur, avoid benzimidazole use;
- if resistance frequency is nil or low, use a maximum of one application per crop per 12-month period;
- confine use to periods when disease risk is high, but disease level is low;
- where practical, apply in a mixture with a protectant fungicide;
- comply with the label application rates at all times;
- practise good crop hygiene and cultural controls;
- using dicarboximides and benzimidazoles in the same spray programme may lead to the development of pathogen strains with dual resistance and should be avoided.

It is recommended that growers who suspect that benzimidazole fungicides fail to control botrytis neck rot in their crops follow the resistance management strategy and send samples for resistance testing. A more intensive monitoring of isolates from all onion-growing areas of New Zealand as well as information on history of spray practices, is required to determine prevalence of carbendazim resistance.

ACKNOWLEDGEMENTS

We thank the seed companies, onion industry personnel and growers who contributed samples and information for this study. MAF Sustainable Farming Fund (grant number 04/110) funded the seed isolate collection. Alpha Research Limited and Technet NZ (contract number CRFX0401) funded the bulb isolate collection and fungicide sensitivity study. Emma Parr provided technical assistance.

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