

In vitro sensitivity testing of *Cladobotryum mycophilum* to carbendazim and prochloraz manganese

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Limited information of fungicide efficacy on cultivated mushrooms and resistance development potential is available. Minor crop industries in general have a smaller arsenal of protectants to rely on and the likelihood of resistance build-up is of greater concern. This study focused on *Cladobotryum mycophilum*'s sensitivity to carbendazim and prochloraz manganese following recent reports on decreased efficacy of both fungicides. The median effective dose (ED₅₀) values for carbendazim ranged between 0.02 mg/L and 4.31 mg/L with 60% of the South African isolates being moderately resistant. The highest resistance factor for carbendazim was 215. Prochloraz manganese ED₅₀ values varied from 0.00001 mg/L to 0.55 mg/L. A significant difference in mean ED₅₀ values for both fungicides tested was observed. Using cluster analysis, no discrimination of isolates previously exposed and unexposed to prochloraz manganese was observed. A wide range of differences in ED₅₀ values indicated moderate resistance to carbendazim and high sensitivity to prochloraz manganese among isolates under investigation. Discriminant analysis indicated significant differences between clusters contributed by one or a few variables. This study provided evidence that prochloraz manganese remains highly fungitoxic to *C. mycophilum*. However, prochloraz manganese is to be used in a disease management strategy in combination with strict farm hygiene management strategies to retain product efficacy and ensure crop protection.

Introduction

Cobweb disease, caused by *Cladobotryum dendroides* (teleomorph *Hypomyces rosellus*) and *Cladobotryum mycophilum* (teleomorph *Hypomyces odoratus*), is one of the three major fungal diseases of the mushroom *Agaricus bisporus* (Lange) Sing.¹ *Cladobotryum mycophilum* is associated with cobweb disease in Australia, the mainland of Europe, the United States of America (USA) and South Africa. *C. mycophilum* is also the dominant species in the British Isles, where it is reportedly resistant to benzimidazoles.^{2,3} *Hypomyces aurantius*, a teleomorph of *Cladobotryum varium* Nees, is another pathogen of mushrooms⁴, whose importance as well, as that of *C. multiseptatum* de Hoog, is not fully understood².

Until the mid 1990s, cobweb disease had not been a major problem in mushroom production in most countries. However, the disease has since become an important obstacle to viable mushroom production in countries such as Australia², the UK and Serbia⁶. It is reported that the disease caused crop losses of up to 40% in 1994/1995 in the Irish and British mushroom industries as a result of serious spotting and early crop termination at the peak of the epidemic.⁵ According to Potočnik et al.⁶ and Back et al.^{7,8}, cobweb disease was first reported on *A. bisporus* in Serbia and Korea in 2007 and 2009, respectively. Sporadic cases of cobweb disease have been reported on several mushroom farms in South Africa during the summer months.

Management of mushroom fungal diseases relies mainly on strict farm hygiene in combination with the use of fungicides.² Although fungicides are used in the control of cobweb disease, there are certain inherent challenges including the fact that both the host and pathogen are fungi.^{9,10} In addition, very few studies on fungicide efficacy on cultivated mushrooms have been conducted and because of its minor crop status, only a few fungicides are currently registered for commercial use.^{11,12} For the South African mushroom industry, only prochloraz manganese, prochloraz zinc² and thiabendazole¹³ are registered for use on mushrooms. Grogan¹⁴ proposed that the mushroom industry's heavy reliance on only two fungicides is a concern for sustainable production. Factors leading to resistance include (1) inherent risk factors relating to fungus biology and fungicide chemistry and (2) management risk factors relating to usage of fungicides in crop management in the wider sense. The inherent factors serve to assess the basic resistance risk for a fungicide/fungus combination in a given area and cannot be controlled. Management of risk factors can be controlled as they are in the hands of farmers, agriculture officials and distributors of fungicides.¹⁵

Benomyl, the first site-specific fungicide to be used in the mushroom industry, has been available from the late 1960s and provides good control of all fungal diseases.¹⁶ Resistance development in *Lecanicillium fungicola* Zare & Gams (syn. *Verticillium fungicola* (Preuss) Hassebrauk and *H. rosellus* was reported in 1974 and 1993, respectively.^{17,18} A few years later, resistance of *Cladobotryum mycophilum* to benzimidazole fungicides was reported in the UK.¹⁹

Prochloraz manganese, one of the demethylation inhibitors (DMIs), was introduced to the mushroom industry in the late 1980s to control wet bubble, dry bubble and cobweb diseases.^{1,20} This fungicide effectively controlled mushroom diseases but over time its effectiveness against *Lecanicillium fungicola* decreased. Since then, increased loss of sensitivity of the pathogen to the fungicide has been reported worldwide.²¹ Prochloraz manganese was the most effective fungicide in the prevention of cobweb disease,¹ but in recent years, it has no longer been able to control the spotting symptoms caused by the disease.¹⁴

Monitoring of fungicide sensitivity within pathogen populations over time is important to ensure more effective management of the disease as well as retention of efficacy of the chemical.^{1,21} Distribution of sensitivity to DMI fungicides among individual strains in an unexposed pathogen population varies from highly sensitive to considerably less sensitive phenotypes. Therefore, exposure to a given DMI fungicide will provide selection pressure favouring the more resistant isolates in the population.^{20,22,23} Progressive reduction in DMI efficacy results

in a practical resistance once the frequency of phenotypes prevents commercially acceptable disease control levels to be achieved under standard usage regimes.²³ The fact that resistance has developed in *L. fungicola*^{21,24} and many other pathogens of field crops,²⁵⁻²⁷ combined with the fact that most *Cladobotryum* spp. produce abundant conidia and have repeated infection cycles, may suggest that *Cladobotryum* spp. might also be at risk.²⁸

We investigated the sensitivity of 32 *Cladobotryum* isolates to prochloraz manganese in order to determine a possible population shift in South African isolates in comparison with isolates obtained from the USA which have not been exposed to this fungicide. The isolates were obtained over a period of time and preserved in a central collection. Carbendazim, although not registered for use on mushrooms in South Africa, but occasionally used, was included in this study to determine its potential fungi toxicity against these isolates.

Materials and methods

Origin and collection of fungal isolates

In total, 32 isolates of *Cladobotryum* spp. were obtained from mushroom farms in South Africa as well as from research institutes around the world (Table 1). Isolates collected from local mushroom farms were either sampled from diseased mushrooms (five) or collected as infected casing (seven), while those from research institutions around the world were received as cultures on agar slants. For each isolate, a single spore culture was prepared for use in this study and preserved in sterile distilled water and in 10% glycerol at -75 °C for short- and medium-term storage for future reference.

Confirmation of isolate identity

To confirm identity of isolates, conidia morphology was examined under a phase contrast microscope (Zeiss, Germany). Colony characteristics were observed on malt extract agar (MEA) (Merck, Johannesburg, South Africa) and potato dextrose agar (PDA) (Merck) upon receipt at the laboratory. Methods for pathogenicity tests were conducted by inoculating a pathogen spore suspension on the pilei of freshly harvested mushrooms according to the Serbian report by Potočnik et al.²⁹ Molecular analysis was conducted to confirm the identity of all isolates through conventional polymerase chain reactions (PCR) using the partial internal transcribed spacer (ITS) region (ITS1 and ITS4) according to Tamm and Pöldmaa³⁰. The DNA sequence was then blasted using the NCBI GenBank database and results were recorded.

Fungicides

Prochloraz manganese (Octave 500 g/kg WP, Bayer Crop Science, Johannesburg, South Africa) and carbendazim (Bendazid 500 g/L EC, Plaaskem, Johannesburg, South Africa) were provided by mushroom farmers. To prepare stock solutions, prochloraz manganese was dissolved in sterile distilled water and then added to molten MEA cooled to approximately 50 °C at the desired test concentration. For carbendazim, the stock solution was added to MEA before sterilisation. For both fungicides, the MEA was poured into 90-mm sterile plastic Petri dishes and allowed to solidify before use.

Fungicide sensitivity test

Mycelial inhibition was assessed by transferring a 5-mm diameter disc taken from the edges of an actively growing single colony culture and placing it inverted in the centre of a plate. To assess the initial in vitro response of mycelial growth to different fungicide concentrations, all isolates were exposed to a concentration range of 1–100 mg/L for both products tested. From these observations, the isolates were grouped into two categories with reference to carbendazim concentration: low concentration (0.1–0.75 mg/L) and high concentration (5–20 mg/L). A series of MEA plates was subsequently prepared with concentrations of 0.0, 0.1, 0.2, 0.35, 0.5 and 0.75 mg/L carbendazim representing the low concentration category and concentrations of 5.0, 10.0, 15.0, 17.0 and 20.0 mg/L carbendazim representing the high concentration category. Concentrations used for the in vitro sensitivity test to prochloraz manganese were 0.0, 1.0, 2.0, 3.5, 4.5 and 5.0 mg/L. For both fungicide

tests, each isolate was tested in triplicate per fungicide concentration and incubated at 25 °C for 96 h. All the media and the fungicide stocks used were prepared as a single batch and used within 24 h per trial. Perpendicular colony diameters were measured every day starting at 48 h of incubation. The experiment was repeated twice.

Table 1: Identity and collection details of isolates used in the study

Isolate code	Year isolated	Species identity (molecular)	Source of origin
IR22	Unknown	<i>C. mycophilum</i>	Ireland
IR23	Unknown	<i>C. dendroides</i>	Ireland
IR24	Unknown	<i>C. mycophilum</i>	Ireland
AU1	2007	<i>C. mycophilum</i>	Australia
AU17	2007	<i>C. mycophilum</i>	Australia
NZ25	2007	<i>C. mycophilum</i>	New Zealand
US20	2003	<i>C. mycophilum</i>	USA
US21	2003	<i>C. mycophilum</i>	USA
US34	2000	<i>C. mycophilum</i>	USA
US35	2000	<i>C. mycophilum</i>	USA
US36	2000	<i>C. mycophilum</i>	USA
US37	2000	<i>C. mycophilum</i>	USA
US40	2000	<i>C. mycophilum</i>	USA
US41	2003	<i>C. mycophilum</i>	USA
US45	2008	<i>C. mycophilum</i>	USA
US46	2008	<i>C. mycophilum</i>	USA
US16	2008	<i>C. mycophilum</i>	USA
US18	2008	<i>C. mycophilum</i>	USA
US19	2008	<i>C. mycophilum</i>	USA
SA29	2010	<i>C. mycophilum</i>	South Africa
SA28	2010	<i>C. mycophilum</i>	South Africa
SA3	2010	<i>C. mycophilum</i>	South Africa
SA26	2010	<i>C. mycophilum</i>	South Africa
SA30	2011	<i>C. mycophilum</i>	South Africa
SA31	2011	<i>C. mycophilum</i>	South Africa
SA32	2011	<i>C. mycophilum</i>	South Africa
SA3	2010	<i>C. mycophilum</i>	South Africa
SA4	2010	<i>C. mycophilum</i>	South Africa
SA27	2011	<i>C. mycophilum</i>	South Africa
SA5	2011	<i>C. mycophilum</i>	South Africa
SA12	2010	<i>C. mycophilum</i>	South Africa
NE33	2011	<i>C. mycophilum</i>	Netherlands

Statistical analysis

The mean effective concentration value (ED_{50}) for each isolate at 96 h was calculated whenever possible by the regressing percentage inhibition

against fungicide concentration. The ED_{50} values of some isolates could not be determined because their growth was too slow. The mean colony diameter for each treatment was expressed as percentage growth inhibition using a SAS general linear model procedure and the means were compared using a *t*-test. Multivariate analysis (Statistica version 10.0) was used to cluster and discriminate between and within groups among isolates tested.

Results

Confirmation of isolate identity and pathogenicity

Conidia of all isolates were 1–3 septate and mostly obvoid in shape except for isolate SA4 which had a few curved conidia. Conidia sizes ranged from 15 to 34 μm long. On PDA, all isolates developed a yellowish colony colour after four days' incubation. On MEA, three distinct colony morphologies were observed in *C. mycophilum*. Colonies with a 'cotton-fluffy' texture were formed by US34, US35, US36, US37 and US41, unlike the more compact or 'felt-like' texture of US40 and IR23 (only *H. rosellus*), an ATCC isolate of Irish origin. The rest of the isolates were 'cotton-like' – neither 'fluffy' nor 'compacted'. Most isolates' colonies were initially yellowish to pinkish, turning purplish after more than 96 h of growth. Blasting of ITS sequence against GenBank gave a 98–99% similarity to *C. mycophilum* for all isolates and IR23 was confirmed as *H. rosellus* (Table 1). These results confirmed the isolates' identity as *Cladobotryum* species.

Pathogenicity of all isolates was confirmed by the development of disease symptoms (cobweb) on the mushroom pilei. The majority (85%) of isolates resulted in cobweb symptom development within 48 h while others, such as *H. rosellus* (IR23) and some *C. mycophilum* (SA3, SA4, SA27 and IR22), were less virulent and took between 72 and 96 h to cause symptoms.

Fungicide bioassays

Most isolates under investigation demonstrated an ability to grow in the presence of bendazid at low concentrations. The ED_{50} values of bendazid against *C. mycophilum* isolates varied between 0.02 mg/L and 4.31 mg/L. The ED_{50} value of a few isolates could not be determined because they were too low. The highest resistance factor (RF) for bendazid was 215.5 mg/L for isolate US21. Isolate SA29 had the highest ED_{50} and RF values although values were not significantly different when compared with other isolates from South Africa.

All isolates tested were highly sensitive to prochloraz manganese and ED_{50} values ranged from 0.00001–0.55 mg/L. Isolates IR23, SA13 and US40 had ED_{50} values less than 0.00001 mg/L and could thus not be determined. Isolate SA12 had the highest RF of 55 000. Only four isolates (AU1, US36, US41 and SA27) had RFs of less than 100. There were significant differences among the isolates under examination. However, none of the isolates showed resistance to prochloraz manganese when their ED_{50} values were compared.

Cluster analysis

Cluster analysis of the isolates based on their response to fungicide treatment tests resulted in 100% classification of six, four and three clusters under prochloraz manganese and carbendazim low and high concentration categories, respectively. Clustering within each treatment was analysed for significant differences using discriminate analysis. For all three treatments, there were significant differences between but not within clusters. Not all variables (test concentrations) contributed to the variations observed.

Cluster analyses of prochloraz manganese treatment results grouped the 32 isolates tested into four clusters (Figure 1). Clustering was done a priori. The largest cluster (IV) included 12 isolates with an average ED_{50} value of 0.33 mg/L. The isolate with the highest ED_{50} value was SA12 (0.55 mg/L) followed by US18 (0.54 mg/L), with RF values of

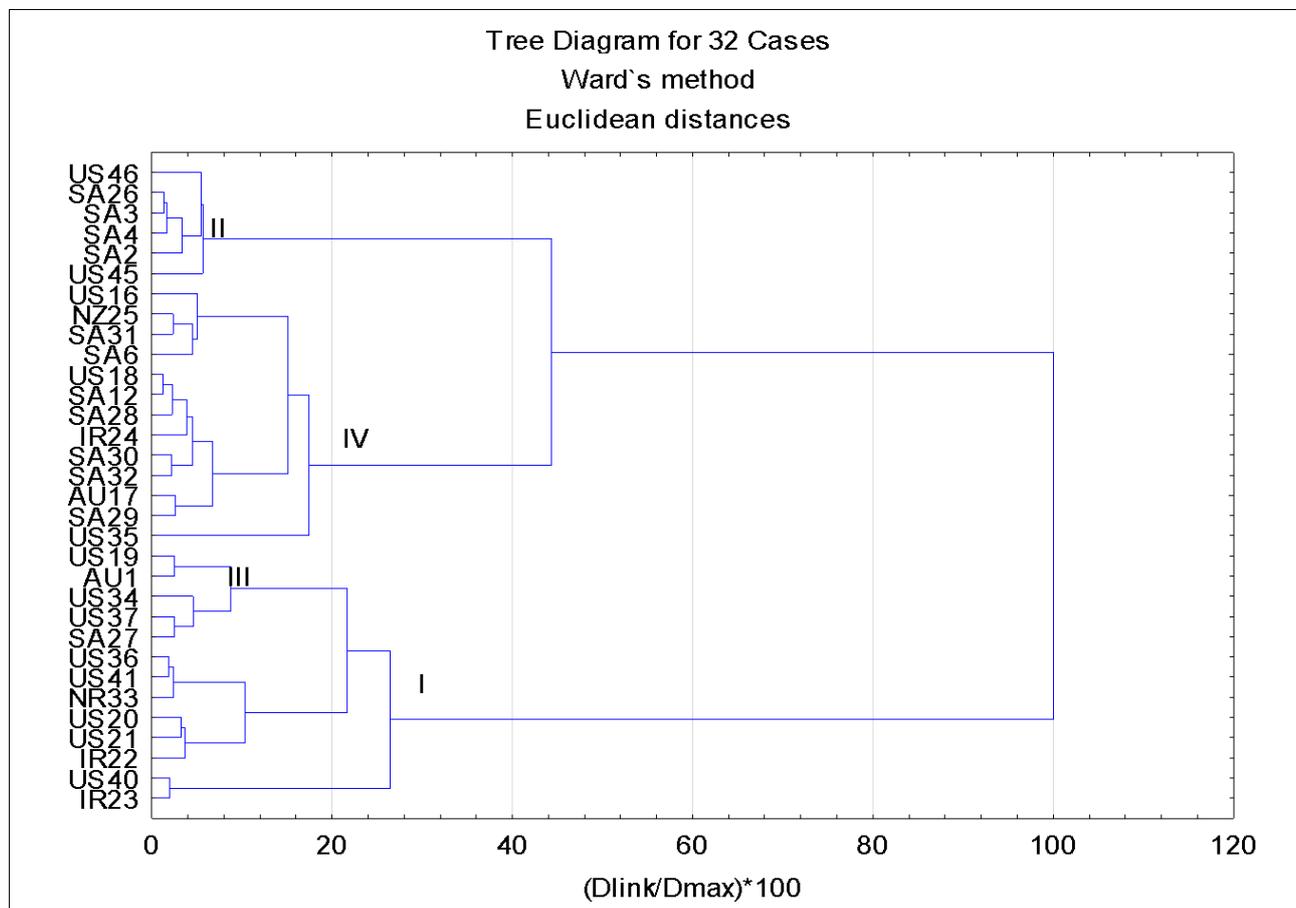


Figure 1: Dendrogram showing clustering of *Cladobotryum mycophilum* isolates into four groups based on prochloraz manganese in vitro sensitivity screening.

55 000 and 54 000, respectively (Table 2). There were no significant differences among the members of this group. The range in ED₅₀ values was between 0.05–0.55 mg/L. South African isolates made up 50% of this cluster, followed by 25% USA; the rest of the isolates were from Australia, Ireland and New Zealand.

Cluster I was the second largest, represented by eight members (IR22, IR23, NR33, US20, US21, US36, US40 and US41) from Ireland, Netherlands and the USA with a mean ED₅₀ value of 0.047 mg/L. The highest ED₅₀ value in the cluster was 0.13 mg/L for IR22, an Irish isolate. Cluster II comprised 71.4% isolates from South Africa (SA26, SA2, SA3, SA4 and SA6) and 28.6% from the USA (US45 and US46). The average ED₅₀ value for Cluster II was 0.23 mg/L with a range of 0.06–0.4 mg/L. The ED₅₀ values of some of the individual members of this group were not significantly different from each other despite the wide range of ED₅₀ values observed (Table 2.). In Cluster III, 60% of the isolates were from the USA, 20% were from Australia and the rest were from South Africa. Cluster III had an average ED₅₀ value of 0.074 mg/L with a range of 0.0002–0.13 mg/L. The highest ED₅₀ value in the group was for isolates from the USA (US19 and US34). The lowest ED₅₀ value was from SA27.

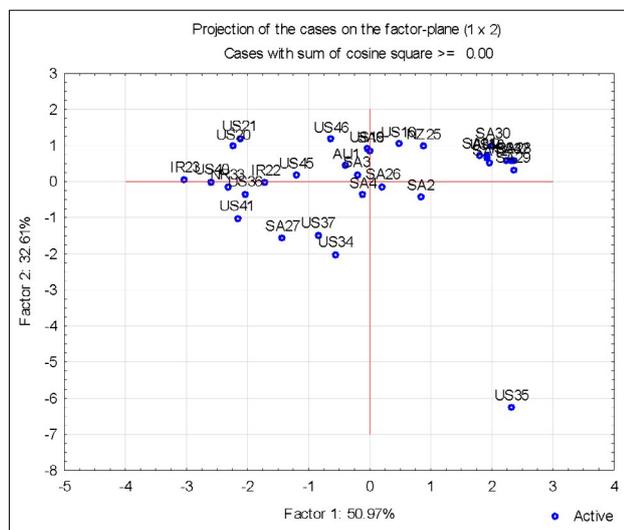


Figure 2: Projection of *Cladobotryum mycophilum* isolates following exposure to prochloraz manganese in vitro sensitivity screening.

The projection of isolates exposed to prochloraz manganese on the plane were for Factors 1 and 2. In Figure 2, eight of the South African isolates are clustered in the higher region of the plane. This shows a tendency of those isolates to tolerate higher concentrations of prochloraz manganese than the rest of the isolates under study.

Cluster analysis for the carbendazim fungicide in vitro test was carried out separately for the low concentrations (ranging from 0.0–0.75 mg/L) and the high concentrations (ranging from 0.0–20.0 mg/L). For easier reference, these are called Analysis 1 and Analysis 2. For Analysis 1, four clusters were formed a priori, with the largest (Cluster III) consisting of six isolates (Figure 3). Four of the isolates in Cluster III were from South Africa and one each was from Ireland and the USA. The average ED₅₀ value for Cluster III was 0.058 mg/L. Isolate US41 was significantly different from the rest of the group. Cluster I had an average ED₅₀ value of less than 0.02 mg/L, which was the lowest of all clusters formed in Analysis 1. Three isolates (US45, IR23 and US34) that were included in this lower concentration category had EC₅₀ values below 0.02 mg/L, the lowest determined value in the category. Cluster II consisted of four US isolates (US35, US36, US37 and US40) and one South African isolate (SA6). Isolates US40 and US37 were significantly different from each other but not from the rest of the cluster. Overall, ED₅₀ values for the isolates exposed to the low carbendazim concentrations ranged from 2.5–10.5 mg/L (Table 3).

For Analysis 2 (carbendazim high concentration), three clusters were also determined a priori (Figure 4). Cluster II with nine isolates had the most members and had an average ED₅₀ value of 3.0–4.31 mg/L.

Table 2: ED₅₀ values indicating the level of sensitivity of *Cladobotryum mycophilum* to prochloraz manganese chloride complex, an imidazole fungicide

Isolate code	^a Mean ED ₅₀	Median ED ₅₀	ED ₅₀ range	^{**} Resistance factor
SA12	0.55 ^a	0.51	0.0076–1.121	55000
US18	0.54 ^{ab}	0.65	0.002–1.005	54000
SA32	0.52 ^{abc}	0.59	0.002–0.983	52000
SA28	0.49 ^{abcd}	0.46	0.001–1.043	49000
IR24	0.43 ^{abcdef}	0.22	0.00003–1.172	43000
US45	0.40 ^{abcdefg}	0.35	0.008–0.883	40000
US46	0.38 ^{abcdefg}	0.18	0.002–1.534	38000
AU17	0.33 ^{abcdefg}	0.32	0.0013–0.666	33000
NZ25	0.16 ^{abcdefg}	0.0085	0.0018–0.727	16000
SA30	0.34 ^{abcdefg}	0.0143	0.002–1.273	34000
SA31	0.15 ^{abcdefg}	0.0087	0.002–0.614	15000
US35	0.31 ^{abcdefg}	0.17	0.0049–0.827	31000
SA6	0.15 ^{abcdefg}	0.18	0.002–0.329	15000
US19	0.13 ^{bcdefg}	0.02	0.00001–0.475	13000
US37	0.13 ^{bcdefg}	0.00013	0.0001–0.536	13000
IR22	0.13 ^{bcdefg}	0.14	0.0002–0.234	13000
US34	0.11 ^{defg}	0.00055	0.003–0.342	11000
SA26	0.09 ^{defg}	0.07	0.00001–0.0140	9000
SA3	0.08 ^{defg}	0.07	0.013–0.185	8000
SA4	0.06 ^{defg}	0.07	0.008–0.111	6000
SA2	0.05 ^{efg}	0.04	0.198–0.103	50000
US20	0.03 ^g	0.012	0.00006–0.153	3000
AU1	0.0007 ^g	0.0006	0.00002–0.002	70
US36	0.00004 ^g	0.00003	0.00001–0.00008	4
US41	0.00001 ^g	0.00001	0.00001–0.00003	3
US21	0.007 ^g	0.005	0.001–0.017	700
SA27	0.0002 ^g	0.002	0.0001–0.0002	20
NE33	0.008 ^g	0.008	0.00001–0.0014	800
SA5	0.002 ^g	0.002	0.0002–0.002	200
IR23	nd	nd	nd	nd
SA13	nd	nd	nd	nd

ED₅₀ is the fungicide concentration that inhibits 50% of fungal growth.

^aMeans followed by different letters are significantly different.

^{**}Expressed as the ratio of the highest ED₅₀ to the lowest ED₅₀ of isolates tested.

nd, not determined.

The two isolates (US21 and SA32) with the highest overall RF values (215.5 mg/L and 207.5 mg/L respectively) belonged to Cluster II. There was no significant difference in the ED₅₀ values of the isolates in Cluster II. Cluster I consisted of all US isolates: US16, US19, US20 and US24. Apart from US16, with an ED₅₀ value of 0.58 mg/L, the ED₅₀ values of

the isolates could not be determined, but they were all lower than that for US16. The average ED_{50} value for this cluster was 0.14 mg/L. Cluster III had only two isolates, AU17 and SA30, for which the ED_{50} values was 2.03 mg/L and 3.95 mg/L, respectively, with an average of 3.0 mg/L.

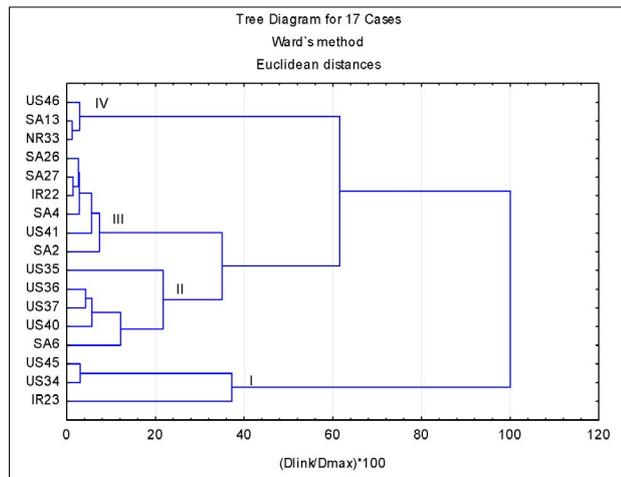


Figure 3: Dendrogram showing clustering of *Cladobotryum mycophilum* isolates into four groups after carbendazim (low concentration) in vitro sensitivity screening.

Discussion and conclusion

A total of 32 *Cladobotryum* isolates was assessed for their sensitivity to prochloraz manganese and carbendazim (Figure 5). Isolates from the USA, where prochloraz manganese is not registered for use in the mushroom industry,³¹ were used as a baseline reference culture for this study. All isolates were sensitive to both carbendazim (0.02–4.31 mg/L) and prochloraz manganese (0.00001–0.55 mg/L). The highest RFs observed with bendazid and prochloraz manganese were 215 (US210) and 5 5000 (SA13), respectively. According to the criteria used by Delp et al.³² and Gouot³³ to define resistance (in which an RF value of

more than 2 is considered resistant), both isolates would have been considered highly resistant to the tested fungicides. The RF provides an indication of the potential for resistance development in a given population. According to the definition by Gea et al.⁹, the higher the RF the greater the difference in sensitivity between the least sensitive individuals in a population relative to the mean sensitivity of the individuals in the entire population and therefore, the greater the potential for resistance to develop.

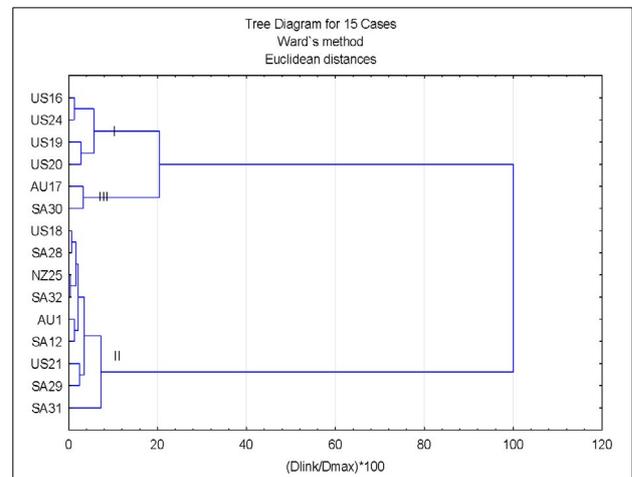


Figure 4: Clustering of *Cladobotryum mycophilum* isolates into four groups after carbendazim high concentration under in vitro treatment.

However, a higher resistance factor value does not imply that resistance will be rapidly expressed in the isolates under study because the ED_{50} values obtained were very low. In this instance, RF values were much higher for both fungicides tested, indicating the likelihood of resistance development for the isolates under investigation.³² ED_{50} and RF values are used as an index to determine resistance or sensitivity of a fungal pathogen to fungicide treatment.¹⁸ According to Potočnik et al.¹⁷ and

Table 3: ED_{50} values indicating the level of sensitivity of *Cladobotryum mycophilum* to carbendazim, a benzimidazole fungicide

Carbendazim low concentration group (mg/L)					Carbendazim high concentration group (mg/L)				
Isolate code	[†] Mean ED_{50}	Median ED_{50}	ED_{50} range	^{**} Resistance factor	Isolate code	ED_{50}	Median ED_{50}	ED_{50} range	^{**} Resistance factor
US34	0.21 ^a	0.20	0.142–0.288	10.5	US16	0.58 ^d	0.58	0.00–0.58	29
US41	0.17 ^{ab}	0.01	0.088–0.300	8.5	SA30	3.95 ^e	3.18	2.84–6.59	197.5
US36	0.16 ^{ab}	0.16	0.158–0.162	8.0	SA31	4.13 ^e	3.84	0.86–6.48	206.5
US35	0.15 ^{ab}	0.15	0.113–0.189	7.5	SA29	4.15 ^e	4.64	2.99–5.03	207.5
SA6	0.16 ^{ab}	0.16	0.099–0.178	8.0	US21	4.31 ^e	4.44	2.79–5.99	215.5
US40	0.12 ^b	0.14	0.088–0.149	6.0	NZ25	3.75 ^e	4.21	1.54–4.98	187.5
US46	0.02 ^c	0.02	0.0001–0.021	1	US18	3.88 ^e	3.75	2.33–5.42	194
SA26	0.05 ^c	0.05	0.0312–0.756	2.5	AU1	3.00 ^{ef}	3.52	0.36–4.65	150
SA27	0.05 ^c	0.05	0.023–0.086	2.5	SA12	3.46 ^{ef}	3.45	2.16–4.91	173
IR22	0.03 ^c	0.03	0.019–0.043	1.5	SA32	3.67 ^{ef}	4.32	1.45–5.21	183.5
SA4	0.05 ^c	0.04	0.021–0.075	2.5	SA28	3.70 ^{ef}	3.76	1.12–5.14	185
AU17	2.03 ^g	1.94	1.05–3.10	101.5	SA5	nd	nd	nd	nd

ED_{50} is the fungicide concentration that inhibits 50% of fungal growth.

[†]Means followed by different letters are significantly different.

^{**}Expressed as the ratio of the highest ED_{50} to the lowest ED_{50} of isolates tested.

nd, not determined.

Gea et al.²¹ a wide range in ED_{50} values indicates a wider range of sensitivity for isolates under investigation. Both Potočnik et al.¹⁷ and Gea et al.²¹ reported that an increase in the range of sensitivity increased the risk of individuals within a population becoming less sensitive to the test fungicide. The gradual selection pressure exerted on the pathogen population can therefore contribute to triggering resistance build-up in *C. mycophilum*. In this study, resistance was not observed in any of the isolates challenged with the two fungicides. However, production practices, such as strict farm hygiene to reduce the incidence of disease, should be maintained to minimise the potential risk of build-up of pathogen resistance.²¹

Among the isolates from the USA, the lowest and highest RFs were 1 and 54 000 with ED_{50} values of 0.00001 mg/L and 0.54 mg/L, respectively. This observation corresponds with the report by Erickson et al.²² indicating that sensitivity to a DMI fungicide among individuals not yet exposed to such a fungicide varies from highly sensitive to more resistant phenotypes. However, in this study, no isolate had an ED_{50} value of less than 5 mg/L or more than 50 mg/L to render them, respectively, moderately or highly resistant according to the criteria set by Russell et al.³⁴ cited by Gea et al.²⁴

The 12 isolates studied from the South African mushroom industry were highly sensitive to prochloraz manganese (ED_{50} =0.0002–0.55 mg/L) and there were significant differences among the isolates under study. However, none of the isolates was resistant to prochloraz manganese. Prochloraz manganese was therefore more toxic than carbendazim to all isolates investigated in the study.

According to Bonnen et al.³¹, the presence of moderately resistant isolates prevalent prior to the benomyl era indicates the existence of a certain level of resistant isolates within the natural population. A number of studies has been conducted to determine sensitivity of mushroom pathogens to prochloraz and benomyl (carbendazim). In the mid-1970s, British researchers conducted a survey in the UK of *Cladobotryum* spp. sensitivity to benomyl.¹⁸ These researchers showed that none of the isolates grew at a benomyl concentration of 5 mg/L. Benomyl has since been replaced by carbendazim; a primary breakdown fungitoxic product of benomyl.³⁵ In their cropping experiment, Fletcher et al.¹ observed that benomyl, thiabendazole and prochloraz manganese controlled *Cladobotryum* spp. satisfactorily. In the Netherlands, it was reported that prochloraz manganese complex was effective against *Hypomyces rosellus*, *Lecanicillium fungicola*, *Mycogone perniciosa* (Magnus) in the cultivation of *A. bisporus* and *A. bitorquis* (Quel.) Sacc.²⁰

In the 1994/1995 outbreak of cobweb disease in the UK, it was reported that *Cladobotryum* spp. isolates were inhibited at a 10 mg/L concentration of carbendazim.³⁵ Resistance of *Cladobotryum* spp. to a benzimidazole fungicide was reported in Ireland in 1992. Further investigations by McKay et al.¹⁹ confirmed that these isolates were resistant to the benzimidazole fungicides benomyl and carbendazim with an ED_{50} of up to 10 mg/L.¹⁹ Grogan and Gaze³⁶ also observed that the majority of the *Cladobotryum* spp. isolated in the UK was strongly resistant to thiabendazole (ED_{50} >200 mg/L) and weakly resistant to carbendazim (3.5–4.4 mg/L).³⁵

Among the isolates in our study, only a third of the isolates were moderately resistant and none were highly resistant. In accordance with the definition by Gea et al.²¹, an ED_{50} of 0–5 mg/L indicates sensitivity, 5–50 mg/L moderate resistance and >50 mg/L resistance – most isolates were sensitive to carbendazim (ED_{50} =0.002–2 mg/L). Of all isolates moderately resistant to carbendazim, 60% were isolates from the South African mushroom farms. Based on Gouot's standard,³³ isolate response to carbendazim exposure (ED_{50} =0.05–4.15 mg/L) ranged from extremely sensitive to moderately resistant among the South African isolates.

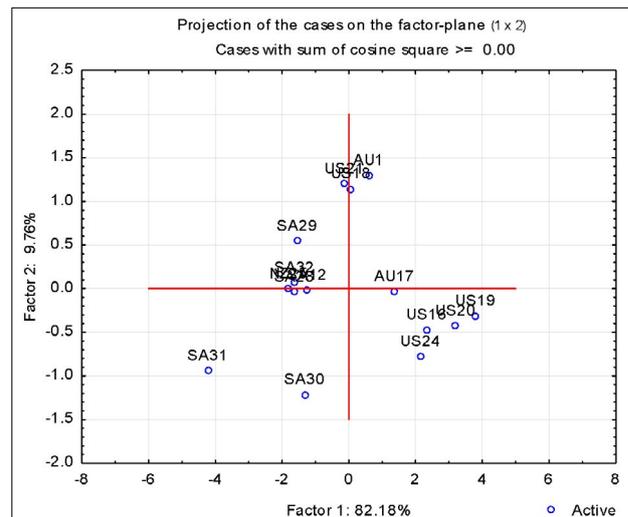


Figure 5: Projection of *Cladobotryum mycophilum* isolates following exposure to carbendazim (high concentration) after in vitro sensitivity screening. Isolates from South Africa did not form defined clusters. Only the four isolates from the USA (US16, US19, US20 and US24) grouped together.

Research findings by Grogan et al.³⁶ showed that isolates in the UK exhibited a range of reactions to prochloraz manganese with ED_{50} values of 0.19–7.80 mg/L. Within their study population, the duo stated that a total of 75% were weakly resistant (1.1–3.7 mg/L) to prochloraz manganese. In another report, Potočnik et al.²⁹ showed that Serbian isolates of *Cladobotryum dendroides* were highly sensitive, with ED_{50} values ranging from 0.01 mg/L to 0.09 mg/L for prochloraz manganese and 0.24–2.92 mg/L for carbendazim.²⁹ In our investigation, none of the isolates showed reduced sensitivity to prochloraz manganese and the chemical remained highly toxic to the isolates under investigation (ED_{50} =0.00001–0.55 mg/L).

From our findings, clustering of isolates using a multivariate analysis did not discriminate between isolates never exposed to prochloraz manganese and those that had been exposed. The earliest (2000) collected isolates received from the USA did not form one cluster, nor was such clustering obtained from the South African isolates. This finding is probably in accordance to observations made by McGath²³ and Erickson et al.²² who proposed that in a population of isolates that have never been exposed to a DMI fungicide, there is a continuously varying range from highly sensitive to highly resistant phenotypes. However, in our study, no isolate had an ED_{50} value of 5 mg/L or more; therefore, no isolate investigated was highly resistant.

Even though the risk of resistance development in the *Cladobotryum* spp. under investigation seemed low, continuous fungicide usage selects for pathogen resistance; thus farm hygiene measures should be managed to reduce incidence of the disease and minimise the risk of build-up of resistance.²⁶ Emergence of benzimidazole resistance reduces the value of these fungicides available for disease control. In addition, a lack of effective alternatives makes it important to manage fungicides with a holistic strategy to retain efficacy. Regular monitoring therefore becomes even more important.¹⁴ In conclusion, mushroom growers can no longer rely exclusively on prochloraz manganese as a primary management tool for fungal diseases.²¹

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Authors' contributions

L.K. was the project leader and promoter and was involved in the experimental design. E.J.v.d.L. was the co-promoter and was responsible for the experimental set-up and for arranging the statistical analyses. Both promoters were also responsible for editing the manuscript. A.C. was responsible for performing most of the experimental work and writing the manuscript.

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