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Resistance of Iranian *Lecanicillium fungicola* to Benzimidazole and Ergosterol Demethylation Inhibiting Fungicides

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ABSTRACT

Dry bubble disease is one of the most important serious diseases of the cultivated white button mushroom (*Agaricus bisporus* (Lange) Imbach). It is a cosmopolitan disease having a worldwide distribution. Ten isolates of *Lecanicillium fungicola* var. *fungicola* (Preuss) Zare and Gams were collected from mushroom farms. Sensitivity of the isolates to benomyl, carbendazim, carbendazim+iprodione and prochloraz manganese were studied. All the isolates were resistant to benomyl(ED50= 415.45-748.12 mg L^{-1}), carbendazim (ED_{50}= 1123.87-1879.59 mg L^{-1}) and iprodione+carbendazim (ED_{50}= 415.45-748.12 mg L^{-1}). However, most of the isolates were sensitive to prochloraz manganese (ED_{50}=1.62–12.58 mg L^{-1}). As the primary source of the pathogen inoculum is casing soil and insects, stringent environmental hygiene of the mushroom houses will play a very important role in preventing and controlling the disease.

Keywords: *Agaricus bisporus*, Dry bubble, Fungicide resistance, Mushroom, Sporgon.

INTRODUCTION

Dry bubble disease is one of the most important serious diseases of cultivated white button mushroom (*Agaricus bisporus* (Lange) Imbach). It is a cosmopolitan disease having a worldwide distribution. The causal agent of the disease is *Lecanicillium fungicola*. The pathogen causes several macroscopic symptoms on its host, including necrotic lesions, stipe blowout and undifferentiated masses called dry bubble (Largeteau and Savoie, 2008).

Although the disease control is maintained by the use of chemicals, there is a limitation in the use of fungicides because both the host and the pathogen are fungi. Currently, Iranian mushroom growing centers rely on the use of prochloraz manganese and benzimidazole groups such as benomyl and carbendazim for control of the disease. The use of fungicides from the group of benzimidazole was prevalent in the late 1960s and in the early 1970s. Also, they were applied for the control of diseases in mushroom farms worldwide. The mechanism of action of benzimidazole is the prohibition of cell division by destruction of beta tubulin of microtubules. A simple shift in amino acids sequence of beta tubulin can prevent adjoining of benzimidazole fungicides to their site of action (Smith, 1988, Chaten, 1996). Benomyl was the first benzimidazole used for mushroom diseases control (Holmes *et al.*, 1971, Snel and Fletcher, 1971). At the first introduction of the fungicide, good control of the disease was observed, but by 1974, several reports demonstrating fungicide resistance were published (Fletcher and Yarham, 1976). In Switzerland, about one year after the benomyl and carbendazim registration for mushroom diseases control, these fungicides lost their ability to control dry bubble disease due to the development of resistance (Gandy and Spencer, 1976). In Australia, benomyl and thiophanate methyl

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resistant isolates were first reported in 1987 (Nair and Macauley, 1987).

In the early 1980s, prochloraz manganese was introduced to mushroom industry for the control of wet bubble disease (*Mycogone perniciosa*), cobweb disease (*Cladobotrium denderoides*) and dry bubble disease (*Lecanicillium fungicola*) (Van Zaayen and Van Adrichem, 1982, Fletcher *et al.*, 1983). Prochloraz manganese belongs to imidazole group and inhibits biosynthesis of ergosterol in cytoplasmic membranes (Buchenauer, 1987). Several reports of the disease resistance to the fungicide already exist. In the study of Geels (1996), the ED_{50} of prochloraz manganese against *L. fungicola* isolates was found to be 40-80 mg L^{-1}. In England, mushroom industry also depends on fungicide application for control of the disease. Grogan *et al.* (2000) reported that values of ED_{50} of this fungicide against *L. fungicola* isolates were in the range of 5-8 mg L^{-1}. The ED_{50} value of the fungicide for one isolate was 5.9 mg $L^{-1}$and it was able to grow on media containing 50 mg $L^{-1}$of this fungicide. The ED_{50} value of prochloraz manganese against *L. fungicola* isolates was determined at 0.8–8.8 mg $L^{-1}$by Gea *et al.* (2005). Formerly, Gea *et al.* (1995) reported that the ED_{50} values of the fungicide against *L. fungicola* isolates were less than 5 mg L^{-1}. However, the ED_{50} determined with the isolates that were collected prior to the fungicide introduction in mushroom farms was 0.7 mg L^{-1} (Gea *et al.*, 2005). In their research, the sensitivity rates of the pathogen to prochloraz manganese decreased gradually from 1996 to 1999.

In the study of Potočnik (2006), all isolates of *L. fungicola* were resistant to benomyl with ED_{50} rates higher than 200 mg L^{-1}, the isolates were of intermediate sensitivity to iprodione, with ED_{50} rates between 11.93–22.80 mg L^{-1}, and were more sensitive to prochloraz manganese with ED_{50} rates lower than 3 mg L^{-1}. The aim of this study was to investigate the sensitivity of ten Iranian isolates of *Lecanicillium fungicola* to benomyl, carbendazim,iprodione+carbendazim and prochloraz manganese.

MATERIALS AND METHODS

Isolation and Identification

The isolates of *Lecanicillium fungicola*, collected from Tehran mushroom growing centers, are presented in Table 1. Isolation was done by cutting small pieces of the diseased mushroom fruit bodies, which were then sterilized by immersion in 1% sodium hypochlorite for one minute. The pieces were placed on potato dextrose agar (PDA) (Potočnik *et al.*, 2008). After isolation of single spores, the isolates were maintained on potato carrot agar (PCA) at 4°C. Isolates were identified based on taxonomic criteria determined by Zare and Gams (2008).

Fungicide Bioassay

Lecanicillium isolates were cultured on potato dextrose agar (PDA) containing the following fungicide: prochloraz manganese (Sporgon WP50 Bayer Crops Co. Germany), carbendazim (Carbendazim WP50 Ghazal Chimi Co. Iran), benomyl (Benomyl WP50 Chimi Keshavarz Iran) and iprodion+carbendazim(Iprodione+Carbendazim WP50 Ghazal Chimi Co. Iran). Suspensions of fungicides in ethanol were added to sterile PDA at 50°C. Fungicide concentrations were expressed based on active ingredient. The concentrations of all selected fungicides were

Table 1. *Lecanicillium fungicola* isolates.

Isolate code	Year of isolation	Origin of isolate
B1	2010	Shahriar
B2	2009	Shahriar
R3	2009	Hashtgerd
G5	2009	Shahriar
G6	2009	Shahriar
G8	2010	Shahriar
F3	2009	Shahriar
F6	2009	Shahriar
P4	2009	Shahriar
P16	2009	Mohammadshahr

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as follow:

Prochloraz manganese: 0.1, 0.5, 1.0, 2.5, 5.0 10.0, 20.0, 50, 100 mg L^{-1}; carbendazim, benomyl and iprodion +carbendazi: 1, 10, 20, 50, 100, 200, 500, 1,000, 1,500, 2,000, 2,500 and 3,000 mg L^{-1}. Five mm mycelial agar disks were used as inoculum and centrally placed on both fungicide amended media and the control media and then incubated at 20°C in total darkness. For each treatment, three replicates were used. Colony diameter minus plug diameter was calculated after 14 days of inoculation and growth of colonies was used as a percentage of the control for ED_{50} calculation i.e. fungicide concentration inhibiting radial mycelia growth by 50%.

The median effective dose (ED_{50}) for each fungicide and isolate was calculated by interpolation from computer-generated log-probit plots (Probit SPSS version 16) of fungicide concentrations and relative growth inhibition: *100 (Colony diameter on amended medium/Colony diameter on control)×100*. The means were separated by the lower and upper bound of each isolate, which were considered as factors in the analysis.

RESULTS

Isolation and Identification

Conidiophores were differentiated from subtending hyphae by being without secondary branches, typically erect, and procumbent with aging. Conidia were produced in globose heads. Octahedral crystals were present, hence all the isolates were identified as *Lecanicillium fungicola*. All the isolates grew at 24°C and no growth was observed at 30°C and, therefore, they belonged to variety *fungicola* (*L. fungicola var fungicola*).

Fungicide Bioassay

The ED_{50} value of benomyl and carbendazim fungicide against *L. fungicola* isolates was varied between 415.45-748.12 mg L^{-1} and 1123.87-1879.59 mg L^{-1}, respectively (Table 2). The ED_{50} values of iprodione+carbendazim and prochloraz manganese against isolates were between 70.88-486.01 mg L^{-1} and 1.62–12.58 mg L^{-1}, respectively (Table 3). For benomyl, iprodione+carbendazim and prochloraz manganese, statistically significant differences were found between the ED_{50} values of different isolates based on the lower and upper bound. None of the isolates were significantly different in their response to carbendazim and none grew on media containing 50 mg L^{-1} prochloraz manganese, except isolate P12. All of the isolates were able to grow on media containing benomyl and carbendazim at concentration of 3,000 mg L^{-1}.

DISCUSSION

The ED_{50} range of benomyl against isolates of *L. fungicola* was between 415.45-748.12 mg L^{-1}. All the isolates were resistant to benomyl. The isolate most resistant to this fungicide was P4 with the ED_{50} of 747.12 mg L^{-1}. Nevertheless, P4 isolate was the most sensitive to carbendazim in comparison to the other isolates.

The ED_{50} ranges of iprodione+carbendazim and prochloraz manganese against the tested isolates were between 70.88-486.01 and 1.62–12.58 mg L^{-1}, respectively. The isolate most resistant to prochloraz manganese was P4, with the ED_{50} of 12.58 mg L^{-1}.

Isolate P4 was different from other isolates in sensitivity to prochloraz manganese, benomyl and carbendazim. This isolate was recognized as one of the most resistant to both benomyl and prochloraz manganese and the most sensitive isolate to carbendazim.

Benzimidazole fungicides were used for the control of mushroom diseases in 1970s for the first time and they controlled diseases effectively early in their introduction (Holmes *et al.*, 1971, Snel and Fletcher, 1971). But, there is a long time since the resistance of *L. fungicola* to carbendazim

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Table 2. ED_{50} values of benomyl and carbendazim for isolates of *Lecanicillium fungicola* var. *fungicola*.

Isolate code	ED_{50}	Lower bound	Upper bound	Slope	*P*-value
			benomyl		
B1	569.71 abc	423.70	801.93	0.72±0.07	0.24
B2	401.32 abc	267.47	699.72	0.73±0.09	0.19
R3	451.68 ab	358.81	577.73	1.04±0.08	0.38
G5	482.61 ab	390.42	607.08	1.03±0.07	0.70
G6	497.89 abc	390.99	650.70	0.89±0.06	0.11
G8	633.88 ac	528.64	769.23	1.31±0.10	0.39
F3	415.45 a	329.31	534.96	1.05±0.11	0.33
F6	682.64 bc	570.51	828.19	1.34±0.10	0.20
P4	748.12 c	617.77	898.78	1.46±0.17	0.19
P16	513.21 abc	409.41	656.36	0.97±0.08	0.23
			carbendazim		
B1	1334.32 a	1180.22	1534.41	2.44±0.31	0.30
B2	1330.13 a	1099.16	1666.21	1.46±0.12	0.36
R3	1818.18 a	1481.17	2418.29	1.58±0.20	0.35
G5	1879.59 a	1353.93	2949.89	0.91±0.10	0.83
G6	1423.84 a	977.36	2358.56	0.69±0.07	0.72
G8	1395.80 a	1126.43	1801.33	1.38±0.12	0.79
F3	1245.70 a	1021.86	1577.10	1.34±0.12	0.92
F6	1285.18 a	955.14	1851.41	0.99±0.08	0.82
P4	1123.87 a	823.92	1661.83	0.79±0.08	0.88
B1	1334.32 a	1180.22	1534.41	2.44±0.31	0.30

ED_{50}= Fungicide concentration which inhibits mycelial growth by 50%, Slope= Regression coefficient at the 95% confidence level.

The ED_{50} followed by different letters are significantly different based on lower and upper bound.

Table 3. ED_{50} values of iprodion+carbendazim and prochloraz manganese for isolates of *Lecanicillium fungicola* var. *fungicola*.

Isolate code	ED_{50}	Lower bound	Upper bound	Slope	*P*-value
			iprodion+carbendazim		
B1	393.99 de	275.79	626.89	0.73±0.08	0.23
B2	270.22 cde	185.01	436.88	0.63±0.09	0.39
R3	486.01 e	354.18	728.35	0.89±0.09	0.36
G5	191.16 bcd	130.33	303.20	0.58±0.07	0.81
G6	70.88 a	44.30	107.59	0.52±0.07	0.64
G8	366.60 de	269.12	539.31	0.84±0.08	0.81
F3	396.48 de	278.31	637.73	0.74±0.10	0.16
F6	109.58 ab	74.76	166.95	0.55±0.05	0.97
P4	202.31 bc	158.80	264.77	0.97±0.07	0.28
P16	130.34 abc	90.93	195.06	0.59±0.06	0.37
			prochloraz manganese		
B1	2.62 abc	2.01	3.75	1.13±0.17	0.69
B2	1.62 a	1.27	2.08	1.25±0.17	0.55
R3	2.77 bc	2.21	3.72	1.37±0.18	0.79
G5	1.98 ab	1.62	2.49	1.47±0.18	0.32
G6	4.75 c	3.46	6.53	0.64±0.05	0.37
G8	2.43 ab	2.00	3.05	1.59±0.19	0.43
F3	2.06 ab	1.62	2.74	1.21±0.17	0.43
F6	2.40 ab	2.02	2.92	1.82±0.19	0.10
P4	12.58 d	9.97	16.17	0.94±0.06	0.93
P16	1.86 ab	1.52	2.32	1.47±0.18	0.667

ED_{50}= Fungicide concentration which inhibits mycelial growth by 50%, Slope= Regression coefficient at the 95% confidence level.

The ED_{50} followed by different letters are significantly different based on lower and upper bound.

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and benomyl was reported (Fletcher and Yarham, 1976; Gandy and Spencer, 1976; Nair and Macauley, 1987).

Gea *et al,* (1996) reported that the values of the ED_{50} of benomyl, chlorotalonil and iprodione against isolates of *L. fungicola* were higher than 50 mg L^{-1}. In a study by Potočnik (2006), all of the isolates were highly resistant to benomyl, with the EC_{50} values exceeding 200 mg L^{-1}, and moderately sensitive to iprodione, having values of EC_{50} between 11.93-22.80 mg L^{-1}. In comparison with these investigations, our bioassay results showed that the Iranian *L. fungicola* isolates were very resistant to benomyl, carbendazim, and iprodione+carbendazim.

Isolates collected from Iranian mushroom growing industry had background of benomyl and carbendazim use for disease control in the cropping system. Thus, cross resistance studies were impossible. Most likely reasons for the appearance of high ED_{50} values of selected fungicides for Iranian *L. fungicola* isolates were attributed to rambunctious use of these fungicides in mushroom industry that caused the population of *L. fungicola* to adapt to these fungicides. All isolates were able to grow on media containing benomyl and carbendazim at 2,000, 2,500 and 3,000 mg L^{-1}, which is in accordance with Gams and Van Zaayen (1982) reports.

Prochloraz manganese was introduced to mushroom industry for dry bubble disease control in late 1980s. In the beginning, it effectively controlled *Agaricus bisporus* diseases. But over time, its effectiveness on the fungus diminished. Lately, decreased sensitivity of the pathogen to this fungicide has been reported worldwide (Geels, 1996; Grogan *et al.*, 2000; Bernardo *et al.*, 2002; Gea *et al.*, 2005; Allan *et al.*, 2008).

In the present investigation, fungicide bioassay results of prochloraz manganese and *Lecanicillium fungicola* isolates showed that most of the isolates were highly sensitive to this fungicide. Among these isolates, isolate P4 with the ED_{50} of 12.58 mg $L^{-1}$was weakly resistant to this fungicide.

Nonetheless, benomyl, carbendazim, and iprodione+carbendazim are still being used for the control of mushroom diseases, especially dry bubble disease in Iran, without any good results. Hence, it is necessary that mushroom growers do not use these fungicides for the control of dry bubble disease. Prochloraz manganese is the only effective fungicide for dry bubble disease control. It is important to decrease the progress of pathogen resistance to this fungicide using integrated disease management items, such as disease monitoring and steam pasteurizing the spent mushrooms substrate after crop termination.

The pathogen shift to a fungicide tolerant population has been proved in several research (Bonnen and Hopkins, 1997; Gea *et al.*, 2005). In the present investigation, fungicides bioassay tests were done on isolates collected recently, but isolates from before introduction of the studied fungicides to Iranian mushroom industry were not available. Therefore, we were not able to confirm population shift to a higher tolerance level to this fungicides.

These information show that the fungicide has gradual selective pressure on the pathogen population and, within some years, the sensitivity rate has been shifted from low to intermediate. In the situation of fungicide use for a given disease control, resistant isolates can better compete with the sensitive ones and the population of isolates resistant to the fungicide increases. Thus, the use of fungicide induces a special selection so that, after some years, there will not be sensitive population of the pathogen and its genetic constitution will be changed to a more clonal population (Largeteau *et al.*, 2006; Largeteau *et al.*, 2008).

The observation of possible failure of Sporgon to control *Agaricus bisporus* dry bubble disease could be a terrible hazardous for Iranian mushroom industry. With the continuation use of this fungicide for the control of dry bubble disease as the single disease control strategy, the pathogen population will be shifted to a more tolerant one to prochloraz-manganese than previous.

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Hence, mushroom growers will not be able to rely on fungicides for dry bubble disease control as the sole tactic in control procedure and should use integrated disease management for further controlling actions.

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مقاومت *Lecanicillium fungicola* ایران به قارچکش‌های گروه بنزیمیدازول و بازدارنده دمتیلاسیون ارگوسترول

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چکیده

بیماری حباب خشک یکی از مهمترین بیماری‌های قارچ خوراکی دکمه‌ای سفید *Agaricus bisporus* (Lange) Imbach به‌حساب می‌آید که شیوع جهانی دارد. ده جدایه از قارچ بیمارگر *Lecanicillium fungicola* var. *fungicola* (Preuss) Zare and Gams از مزارع پرورش قارچ خوراکی جمع‌آوری گردیده و حساسیت این جدایه‌ها به بنومیل، کاربندازیم، ایپردیون+کاربندازیم و پروکلراز منگنز مورد مطالعه قرار گرفت. همه جدایه‌ها به بنومیل (mg/L ED_{50}=۴۱۵/۴۵-۷۴۸/۱۲)، کاربندازیم (mg/L ED_{50}=۱۱۲۳/۸۷-۱۸۷۹/۵۹) و ایپردیون+کاربندازیم (mg/L ED_{50}=۷۰/۸۸-۴۸۶/۰۱) مقاوم بودند. بیشتر جدایه‌ها به پروکلراز منگنز حساس بودند (mg/L ED_{50}=۱/۶۲-۱۲/۵۸). همانگونه که منبع اولیه قارچ بیمارگر خاک پوششی و حشرات است، پاستوریزاسیون صحیح در کنار اقدامات بهداشت محیط و سالن‌های پرورش قارچ خوراکی نقش مهمی را در جلوگیری و کنترل بیماری ایفا می‌کند.

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