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Toxicity of biofungicide Timorex 66 EC to *Cladobotryum dendroides* and *Agaricus bisporus*

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ABSTRACT

Twenty *Cladobotryum dendroides* isolates from Serbian *Agaricus bisporus* farms collected during 2003–2007, and the F56 strain of *A. bisporus* were tested *in vitro* for sensitivity to tea tree oil (Timorex 66 EC), a biofungicide, in comparison with prochloraz–manganese (Octave WP). The efficacies of tea tree oil and prochloraz–manganese were evaluated in a mushroom growing room, after application at standard product application rates and a combination of the two at respective proportion of 20:80%. Tea tree oil was considerably less toxic than prochloraz–manganese *in vitro* to *C. dendroides* isolates (ED_{50} 112.9–335.8 mg l^{-1}) and *A. bisporus* F56 strain (98.0 mg l^{-1}), although neither fungicide was lethal to the pathogen. The biological efficiency of tea tree oil was higher than in treatments with the reference formulation of prochloraz–manganese. In our *in vivo* trials, no negative interference of the biofungicide with *A. bisporus* physiology was observed. Tea tree oil applied at the standard product application rate caused a significant reduction in cobweb disease levels in the *A. bisporus* growing room. Timorex 66 EC should be tested further in combination with other biofungicides to investigate the effectiveness of various mixtures for *A. bisporus* disease control.

1. Introduction

Many compounds have been tested as control agents against edible mushroom diseases. Plant extracts, essential oils and their components have demonstrated strong fungistatic effects (Džamić et al., 2008; Glamočlija et al., 2008; Soković et al., 2009). Although fungitoxic activity of oils is not strong, they could be used as a supplement to commercial preparations for disease control, which will minimize the quantity of fungicides used.

Cobweb disease, caused by *Cladobotryum dendroides* (Bull.: Fr.) W. Gams & Hooz. (teleomorph *Hypomyces rosellus* (Alb. & Schwein.: Fr.) Tul.), is one of the most serious diseases of *Agaricus bisporus* (Lange) Imbach in Serbia (Potočnik et al., 2009). It has been controlled by prochloraz, which is officially recommended for mushroom cultivation in EU countries (Grogan, 2008). Mycopathogen resistance to benomyl, carbendazim and thiabendazole, (Gaze, 1995; McKay et al., 1998; Grogan and Gaze, 2000), and increasing tolerance to prochloraz (Gea et al., 2003, 2005; Grogan, 2006) have already been reported. However, with regard to fungicide resistance evaluation and consumers' demand for more restrictive pesticide use, special attention should be focused on developing alternative approaches in disease management strategies.

Tea tree oil is an essential oil obtained by steam distillation of the Australian plant *Melaleuca alternifolia* (Maiden. & Betche.) Cheel. It contains over 100 components, mostly monoterpenes, sesquiterpenes and their alcohols (Brophy et al., 1989). The main active components of tea tree oil are terpinen-4-ol (42%), α-terpineol (3%) and 1.8 cineole (2%) (Hart et al., 2000). The oil is an effective antiseptic, fungicide and bactericide (Carson et al., 2006). A new formulation, Timorex 66 EC, containing 66% of tea tree oil, is effective against a broad spectrum of pathogens in vegetables, herbs, field crops, fruit trees and grapevines, without causing phytotoxic effects (Reuveni et al., 2006). The mode of action of tea tree oil is not clearly understood, but it acts as a protector against a wide range of fungi by inhibiting spore germination and mycelia growth, while it does no harm to natural enemies and other beneficial insects. Timorex 66 EC has been tested as a biofungicide against phytopathogens but tests against mycopathogens have been scarce (Reuveni et al., 2006).

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The objective of this study was to evaluate *in vitro* the sensitivity of *C. dendroides* isolates originating from Serbia and the F56 strain of *A. bisporus* to tea tree oil, and its biological efficacy in controlling cobweb disease in a mushroom growing room.

2. Material and methods

2.1. Fungi

Isolates of *C. dendroides* obtained from diseased fruiting bodies of *A. bisporus* with symptoms resembling cobweb disease were collected in Serbia during 2003–2007. Conidia of all isolates had one to three septa, with conspicuous basal hilum and phyalide extensions. Camphor-like odor was absent. The radial growth rates of the studied isolates ranged between 11.6 and 14.5 mm day^{-1} on Malt Extract Agar (MEA) at 25 °C. Chlamidospores and microsclerotia were present. Serbian *C. dendroides* isolates were weakly resistant to thiophanate-methyl (EC_{50} 6.5–12.1 mg l^{-1}) and sensitive to carbendazim and prochloraz–manganese (Potočnik et al., 2009). Identification was consistent with previous studies (McKay et al., 1999; Grogan, 2006). Twenty Serbian *C. dendroides* isolates (Table 1) and a culture of *A. bisporus* strain F56 (Italspawn, Italy) were used in this study. The cultures were kept on Potato Dextrose Agar (PDA), at 5 °C, in a culture collection of the Institute of Pesticides and Environmental Protection, Belgrade.

2.2. Crop protection products

Commercial fungicide formulations were used in this study. The organic biofungicide tea tree oil (Timorex 66 EC, Stockton Chemical Corporation, USA, containing: tea tree oil 66%, NaOH 2.72%, inert ingredient 31.28%) was tested as a potential antifungal agent against Serbian *C. dendroides* isolates *in vitro* and in a mushroom growing room. Oil toxicity was also tested to mycelial growth of *A. bisporus* strain F56. The efficacy of tea tree oil was evaluated in comparison with that of the commercial fungicide prochloraz–manganse (Octave WP, Bayer Crop Science, Germany, prochloraz–manganese complex 50%, kaolin 35%, other ingredients 15%).

Table 1
List of *Cladobotryum dendroides* isolates from Serbia and their sensitivity to tea tree oil.

Isolate code	Origin of isolate	Year of collection	ED_{50}[a]			
			Mean	Range	Slope	Range
SP_1C_4	Smederevska Palanka	2003	129.7	107.4–157.8	2.0	1.8–1.2
P_3C_1	Pozarevac	2003	145.1	119.8–173.7	1.8	0.7–2.0
Ba_1C_1	Banjica	2004	199.8	164.1–250.3	1.9	1.7–2.1
B_1C_1	Beograd	2004	288.1	75.3–700.3	1.9	1.7–2.1
Ku_1C_1	Kurjace	2004	160.6	133.3–196.7	2.0	1.8–1.2
NSl_1C_1	Novi Slankamen	2004	224.3	90.2–558.4	2.0	1.8–1.1
OB_1C_2	Ovcar Banja	2004	185.9	154.6–230.1	2.5	2.3–2.8
OB_1C_3	Ovcar Banja	2004	157.3	128.5–191.5	1.7	1.6–1.9
P_7C_1	Pozarevac	2004	262.5	124.4–545.4	2.5	2.4–2.7
Res_1C_1	Resnik	2004	335.8	265.5–434.3	1.3	1.2–1.5
VG_3C_2	Vracev Gaj	2004	185.8	147.4–242.7	1.5	1.4–1.7
Bec_1C_1	Becej	2006	112.9	89.2–141.8	1.6	1.4–1.8
Jak_1C_1	Jakovo	2006	209.9	69.8–612.4	2.1	2.0–2.3
Kal_1C_1	Kaludjerica	2006	139.1	111.0–176.3	1.6	1.4–1.8
NSl_2C_1	Novi Slankamen	2006	116.6	89.2–151.0	1.4	1.3–1.6
Bec_2C_1	Becej	2007	135.2	106.1–168.3	1.5	1.3–1.6
Veg_2C_1	Veliko Gradiste	2007	207.4	169.9–254.8	1.8	1.6–1.9
VG_2C_2	Vracev Gaj	2007	157.6	128.2–197.4	1.8	1.6–2.0
NSl_3C_1	Novi Slankamen	2007	225.2	173.5–312.5	1.4	1.2–1.6
NSl_4C_1	Novi Slankamen	2007	188.7	117.6–340.6	1.6	1.2–1.6

[a] ED_{50} expressed as mg l^{-1} of fungicide.

2.3. In vitro tests

C. dendroides isolates and *A. bisporus* strain F56 were grown on PDA amended with tea tree oil. A 1% tea tree oil stock emulsion was prepared. Oil dispersion was enhanced by adding a Tween 20 (v/v 0.1%) (REANAL Finomvegyszergyar Rt., Hungary, No.: 805383) (Papadopoulos et al., 2006). The selected volumes of biofungicide stock emulsion were added to molten sterile medium (50 °C) to provide concentrations of tea tree oil ranging between 0.01 and 1000 mg l^{-1} (Faleiro et al., 2005). The selected concentrations of tea tree oil were: 10, 50, 100, 500, 1000 mg l^{-1} for *C. dendroides* isolates and 0.1, 1, 10, 100, 500 mg l^{-1} for *A. bisporus* strain F56. Each plate was inoculated with an inverted mycelium agar disc (10 mm), taken from the edge of 4-day old cultures of *C. dendroides* isolates and 20-day old culture of *A. bisporus* F56, placed centrally onto the fungicide-amended media and incubated at 20 °C. Colony diameter was measured after three days of cultivation of *C. dendroides* and 20 days of *A. bisporus*. Mycelial growth on the fungicide-amended medium was presented as a percentage of control. The fungicide concentration which inhibited mycelial growth by 50% (ED_{50}) was defined for each isolate (Grogan and Gaze, 2000). Data on fungicide concentrations and relative inhibition were analysed using probit analysis, according to Finney (1971). Three replicates per treatment and isolate were used to enable statistical analysis. The selectivity index for each active ingredient was calculated as a ratio of ED_{50} mean value for the tested *C. dendroides* isolates and the corresponding single estimate for *A. bisporus* F56 (Chrysayi-Tokousbalides et al., 2007).

Agar dilution minimum inhibitory concentrations (MIC) tests were performed using standard methods (Ishii, 1995). PDA with tea tree oil incorporated at concentrations from 0.98 to 1000 mg l^{-1} was applied in doubling dilutions. Plates were inoculated with an inverted mycelium agar disc (10 mm), taken from the edge of 4-day old cultures of *C. dendroides* isolates and incubated at 20 °C. The lowest concentration of tea tree oil showing no growth after 7, 14 and 21 days was read as MIC. To determine minimum fungicide concentration (MFC), the mycelium agar discs were removed from the fungicide-amended media and placed on surfaces of fungicide-free agar. The plates were incubated for 7 days at 20 °C and the MFC was defined as the lowest concentration of tea tree oil at which 95% lethal effect of the inoculums was determined by absence of growth.

2.4. Efficacy of fungicides in mushroom growing room

A *C. dendroides* isolate, strain OB_1C_2, was grown on PDA at 20 °C for four days. Conidia were harvested by flooding the plates with 10 ml of sterile distilled water and Tween 20 (v/v 0.01 %) followed by filtration through double layers of cheesecloth. Conidia concentration was set at 1000 conidia/m^2 and was used for in *in vivo* experiments in a mushroom growing room.

Plastic bags, 0.60 × 0.40 × 0.25 m ($l \times w \times h$), filled with 18 kg of compost spawned with *A. bisporus* F56, produced by Uča & Co. (Vranovo, Serbia), were incubated (spawn-run) for 18 days at 24 °C. Compost surface was divided by wooden barriers into six sections so that each experimental compartment, measuring 0.20 × 0.20 × 0.25 m, had a total area of 0.04 m^2, and contained 3 kg of spawned substrate. Each plot was cased with a 40-50 mm layer of black peat/lime casing soil (Makadam Co., Belgrade, Serbia) and incubated at 21 °C for 8 days (case-run), and then air temeperature was reduced to 16 °C. Drench applications of tea tree oil and prochloraz–manganese were applied on day 6, and relevant plots inoculated with conidia suspension of *C. dendroides* isolates 9 days after casing. The inoculated and uninoculated bags were treated with: (i) prochloraz–manganese at standard product