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Residue Evaluation of the Azole Fungicides Prochloraz and Tebuconazole in the White Mushroom *Agaricus bisporus*

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Chemical control of diseases of cultivated mushrooms is based on a small number of fungicides due to the limited availability of data supporting registration of suitable formulations. Also in many countries the occurrence of resistant strains to benzimidazoles (Bonnen and Hopkins 1997) has additionally reduced the number of available effective fungicides against serious diseases in mushrooms such as dry bubble disease of *Agaricus bisporus* caused by *Verticillium fungicola*. Currently, chemical control of the disease is based on a few fungicides and the only plant protection products still officially recommended in EU countries are formulations of carbendazim, prochloraz, prochloraz-manganese complex and chlorothanonil (Fletcher et al. 1989; Gea et al. 1995; Whitehead 2002; Index Phytosanitaire 2005; https://secure.pesticides.gov.uk/pestreg/).
In a project of evaluating azole fungicides against *V. fungicola* on *A. bisporus* the performance regarding efficacy and dissipation of the sterol biosynthesis inhibitor tebuconazole was studied in comparison with prochloraz which shares the same subcellular target. The results from residue analysis and relevant dietary risk assessment are presented in this report.

MATERIALS AND METHODS

Trials were conducted in cultures of the commercial strains 2810 (Trial I) and X22 (Trials II, III, IV) of *A. bisporus* in mushroom growing rooms in accordance with the real practice.

The fungicides used for the determination of the residues were of analytical grade. Tebuconazole [(RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol] and prochloraz (N-propyl-N[2-(2,4,6-trichlorophenoxy)ethyl]imidazole-1-carboxamide) were kindly provided by Bayer CropScience, Germany and Aventis CropSciences, Germany, respectively. The commercial formulations Folicur 25 WG (tebuconazole 25%) and Octave 50 WP (prochloraz 50%) were used in the trials.

Plastic boxes measuring 0.40 x 0.36 x 0.19 m were filled with 12-14 kg of *A. bisporus* spawned compost with a moisture content of 70-72% fresh weight. The spawn was prepared on sterilized millet seeds and mixed with compost at a rate of

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2% w/w. The boxes were placed in mushroom growing rooms under controlled conditions arranged in a completely randomized block design. After 13 days of incubation at 26 °C and a relative humidity of 70% a 4-cm thick casing layer containing black peat and limestone (BVB, Euroveen) was added. The boxes were kept at 24.5-26.5 °C for 5-6 days and then fresh air was introduced until a temperature of 17 °C and a CO_2 concentration of 0.09-0.12% (v/v) were reached. Temperature of 17.5±0.5 °C and a relative humidity of 85-90% were maintained throughout cropping. Irrigation of the cultures started when sporophores had reached the "pea" size stage. In the first two trials the fungicide suspensions in tap water were added as single drench applications to the material in the boxes 5 days after casing. Two litres of each preparation were added to the boxes at a rate of 0.8-1.8 g of active ingredient per m^2 of mushroom bed. In the third and fourth trials prochloraz was used as single applications but also as split applications 5 days after casing and 20 days later.

Mushroom samples (0.5 kg/treatment) were taken on the day of the peak production of each of three consecutive flushes. Then the fresh mushrooms were cut into shreds (2-3 cm x 1-2 mm) and stored at -21 °C until analyzed. The size of each lab sample was 10 g.

For prochloraz extraction, each lab sample was extracted with acetone (100 mL) on a Thermolyne Big Bill SE horizontal shaker for 10 minutes at 400 rpm. The extract was then filtered through Whatman No.1 filter paper and the cake was washed with 20 mL of acetone. Then 5 mL of 0.25 M HCl was added to the combined extracts and the acetone was removed under vacuum at 37 °C using a rotary evaporator (Buchi R-3000). The remaining aqueous solution was transferred to a separatory funnel containing 72.5 mL of 0.25 M HCl and extracted with 10 mL of hexane which was then discarded. The pH of the remaining solution was adjusted with sodium hydrogen carbonate (8 g) to approximately 6.7, the solution returned to the separatory funnel and extracted with hexane (2 x 25 mL). The organic phase was passed through a Whatman No.1 filter paper containing 10 g of anhydrous sodium sulphate, the filter cake was then rinsed with 20 mL of hexane and the combined extracts carefully brought to dryness on a rotary evaporator under vacuum. The residue was redissolved in acetone to a volume of 1mL and stored at -18 °C until GC analysis.

For tebuconazole extraction, each lab sample was extracted using 30 mL of acetone on a horizontal shaker (400 rpm) for 3 minutes. Celite 545 (1.5 g) was then added and the mixture was filtered through a Whatman No.1 filter paper. The homogenized preparation was rinsed twice with 10 mL of acetone:water (3:1) mixture and the combined filtrates were transferred to a separatory funnel. Then sodium chloride (4 g) was added and the solution was extracted with 10 mL of dichloromethane. The organic phase was collected, the aqueous part was extracted with 25 mL of dichloromethane and at the end the organic fractions were combined. Anhydrous sodium sulphate (10 g) was added to the organic extract which was then passed through a layer of anhydrous sodium sulphate 3-cm deep on a Whatman No.1 filter paper. The flask and filter cake were then rinsed with

dichloromethane (3 x 25 mL) and the filtrates were combined. The resulting organic extract was carefully brought to dryness on a rotary evaporator under vacuum at 37 °C and the residue was dissolved in 1 mL of ethyl acetate and stored at -18 °C until GC analysis.

A Hewlett-Packard 5890 Series II gas chromatograph equipped with an electron capture detector (packed inlet) and a mass spectrum detector (HP 5971A) were used for residue determination. Injections (2 μL) were made with an autosampler (HP 7673) using the HP 3365 Chemstation Software for instrument control and data acquisition and analysis. For ECD, a 30 m x 0.53 mm capillary column coated with a 1.00 μm thick film of 5% diphenyl–95% dimethyl polysiloxane (Restek RTX-5) was used, with helium as a carrier gas at a flow rate of 6.5 mL min^{-1}. For detection using MS the inlet was operated in the splitless mode and a 25 m x 0.25 mm capillary column with a 0.25 μm thick film (Restek RTX-5) was used with helium as a carrier gas at a flow rate of 1 mL/min. The MS detector was operated in the selective ion mode and ions of 83 and 125 m/z were monitored. For prochloraz analysis the injector and detector temperatures were maintained at 200 °C and 300 °C, respectively. The oven temperature was initially maintained at 120 °C for 3 minutes, then programmed at 15 °C/min to 200 °C with hold time of 2 minutes and finally at 5 °C/min to 280 °C with hold time of 10 minutes. For tebuconazole analysis the injector and detector temperatures were maintained at 250 °C and 300 °C, respectively. The oven temperature was initially maintained at 100 °C for 1 minute, then programmed at 20 °C/min to 280 °C with hold time of 3 minutes. The retention times of prochloraz and tebuconazole were 21.2 and 12.0 minutes, respectively.

Dietary risk assessment of the potential chronic intake was carried out using consumption data included in a relevant publication of the World Health Organization (GEMS 2003).

RESULTS AND DISCUSSION

The pro-analysis quality of the solvents was found to be free from interferences. The calibration curves prepared for each analyte were linear over the range determined with $r^2 \geq 0.99$. Fortifications were made at the level of the residues detected in the mushroom samples. Recovery percentages were within the acceptable range (70-110%) with relative standard deviations (RSDs) lower than 20% (Table 1).

Following application of prochloraz at the rate of 1.2 g/sq m, the residues determined in all mushroom samples were below 0.0250 mg/kg. Only when the fungicide was drenched as split applications, with the second application 25 days after casing were the residue concentrations in the harvested mushrooms twice as much (Table 2). Although all estimated residue values are much lower than 2 mg/kg which is the MRL set by both the European Union (Commission Directive 2002/79/EC) and the Codex Alimentarius Committee on Pesticide Residues in Food (http://faostat.fao.org/faostat/collections?subset=FoodQuality) for

Table 1. Recovery of the analytical methods used for residue determination of prochloraz and tebuconazole.

Analyte	Fortification level (mg/kg)	Recovery (%)	SD (%)	RSD (100% x mean/SD)
prochloraz	0.002	102	15.1	14.7
	0.020	100	3.7	3.7
	0.200	99	2.7	2.8
tebuconazole	0.0020	102	9.4	9.0

Table 2. Prochloraz residues in sporophores of *Agaricus bisporus* in each of three consecutive production flushes (F1, F2, F3) in four trials (I, II, III, IV).

Treatment[a] (g/sq m)	Residues ± SD (mg/kg)											
	I			II			III			IV		
	F1	F2	F3	F1	F2	F3	F1	F2	F3	F1	F2	F3
prochloraz 1.2	0.0250 ±0.0035	0.0226 ±0.0028	<0.0140	0.0018 ±0.0002	0.0181 ±0.0038	0.0250 ±0.0080	0.0038 ±0.0006	0.0049 ±0.0005	0.0082 ±0.0007	0.0063 ±0.0012	0.0065 ±0.0014	0.0091 ±0.0012
prochloraz 1.8	nt[b]	nt	nt	0.0066 ±0.0019	0.0153 ±0.0043	0.0121 ±0.0028	nt	nt	nt	nt	nt	nt
prochloraz 0.6/0.6	nt	nt	nt	nt	nt	nt	0.0238 ±0.0031	0.0551 ±0.0075	0.0116 ±0.0010	0.0028 ±0.0006	0.0633 ±0.0218	0.0160 ±0.0030

[a]Prochloraz formulation was used as single drench application 5 days after casing or as split application (slash) 5 days after casing and 20 days later. [b]nt: non-tested

Table 3. Dietary risk assessment of the potential chronic intake of tebuconazole.

Commodity	Consumption[a] (g/person/day)	Residues (mg/kg)	Theoretical Maximum Daily Intake	
			mg/kg bw/day	% of ADI
mushrooms	4.0	0.01[c]	0.00000067	0.0017
banana[b]	22.8	0.05[d]	0.00001900	0.0475
barley[b]	19.8	0.2[d]	0.00006600	0.1650
cherries[b]	3.0	5[d]	0.00025000	0.6250
cucumber[b]	7.0	0.2[d]	0.00002333	0.0583
grapes[b]	13.8	2[d]	0.00046000	1.1500
oats[b]	2.0	0.05[d]	0.000002	0.0042
peach[b]	12.5	1[d]	0.000208	0.5208
peanut[b]	3.0	0.05[d]	0.000003	0.0063
pepper[b]	10.4	0.5[d]	0.000087	0.2167
pome fruits[b]	51.3	0.5[d]	0.000428	1.0688
rye[b]	1.5	0.05[d]	0.000001	0.0031
squash[b]	3.5	0.02[d]	0.000001	0.0029
tomato[b]	66.6	0.2[d]	0.000222	0.5550
wheat[b]	178	0.05[d]	0.000148	0.3708
Total			0.001918	4.796

[a]by a European adult of 60 kg body weight. [b]commodities for which Codex MRLs have been set. [c]highest value from four residue trials. [d]Codex MRLs

prochloraz in mushrooms, it is important to point out that as far as residues in the produce are concerned the data favour one application of this fungicide close to casing. Supportive data regarding low levels of prochloraz residues in mushrooms have also been obtained from a recent monitoring surveillance in Northern Ireland (Mitchell and Kilpatrick 2003). Residue studies of prochloraz-manganese have shown that the fungicide is of low persistence in casing soil (Grogan and Jukes 2003). The consumer exposure to highest determined residues of 0.0663 mg/kg (Table 2) via consumption of mushrooms treated with prochloraz are below the calculated exposure based on the established EU MRL of 2 mg/kg (Commission Directive 2002/79/EC). Therefore the potential chronic and acute dietary risks could be considered toxicologically acceptable.

It has been reported that the half life of tebuconazole in soil is 49 days or much longer (Strichland et al. 2004; www.pesticideinfo.org) which indicates that the fungicide might remain in mushroom casing soil throughout the entire cropping period. Interestingly, tebuconazole residues in sporophores in all three harvests following single application of the fungicide at 0.8 and 1.2 g/sq m 5 days after casing were below 0.01 mg/ kg, which was the limit of quantification of the method used. EU MRLs have not been established but MRLs and US Tolerances have been set by Codex Alimentarius Committee on Pesticide Residues in Food (http://faostat.fao.org/faostat/collections?subset=FoodQuality) and Code of Federal Regulation (e-CFR 2004), respectively. The Acceptable Daily Intake (ADI) of tebuconazole has been set at 0.03 mg/kg bw/day but an Acute Reference Dose (ARfD) has not been allocated due to low acute toxicity of the fungicide (JMPR 1994). The calculated Theoretical Maximum Daily Intake (TMDI) shows

that the consumer exposure to tebuconazole residues via consumption of mushrooms treated with 1.8 g/sq m is below the ADI (Table 3) and the potential chronic risk could be considered toxicologically acceptable. Furthermore, acute risk could not be estimated due to the low acute toxicity of the fungicide.

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