Pesticides in Fermentative Processes of Wine

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The influence of six fungicides (azoxystrobin, cyprodinil, fludioxonil, mepanipyrim, pyrimethanil, and tetraconazole) on the fermentative activity of two yeasts (*Saccharomyces cerevisiae* and *Kloeckera apiculata*) and two lactic bacteria (*Leuconostoc oenos* and *Lactobacillus plantarum*) was studied. The possibility of their being degraded by these yeasts and bacteria was also investigated. The presence of the pesticides did not affect alcoholic fermentation, not even with levels higher than those normally found in grapes in field experiments. On the contrary, their presence stimulated the yeast, especially *K. apiculata*, to produce more alcohol. The fermentative process did not affect the amount of pesticides either by degradation or by adsorption. During malolactic fermentation by *Le. oenos*, malic acid decreased slightly less (by ∼15%) in the presence of all pesticides, except mepanipyrim. A lower effect (∼5%) was found during the fermentative process with *La. plantarum*. The bacteria studied did not show a degradative effect on pesticides during malolactic fermentation.

Keywords: *Pesticides; yeast; bacteria; alcoholic fermentation; malolactic fermentation*

INTRODUCTION

The production cycle of wine includes two fermentative processes: the alcoholic and the malolatic fermentations. The former transforms sugars into alcohol by yeast, the latter malic acid into lactic acid by bacteria. The presence of pesticides could affect the activity of these microorganisms. Folpet and Captan showed a high antiseptic activity against yeast (Cabras et al., 1987, and literature cited therein). Captafol and dichlofluanid, which were introduced in the market later, showed a similar behavior. A delay in fermentation was also observed with thiophanate methyl (Gaia et al., 1978) and fenarimol (Zironi et al., 1991). For these reasons, at the registration of new pesticides today, manufacturers must show that the products are completely inactive against fermentative microflora. The activity of pesticides against lactic bacteria has been studied less, and no work has been reported on the negative effects of bacteria during malolactic fermentation.

If on the one hand pesticides can affect the activity of yeasts, on the other yeasts can decrease pesticide residues. Studies concerning a large number of classes of pesticides showed that yeasts can decrease the amount of pesticides by degradation and adsorption (Cabras et al., 1995, 1997, 1998; Farris et al., 1989; Fatichenti et al., 1983, 1984). The degradation of pesticides is also caused by bacteria (Cabras et al., 1994). A lot of studies have clarified the interaction between pesticides, yeasts, and bacteria. No information is available for last-generation pesticides. The aim of this work is to contribute to a better knowledge on this subject.

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MATERIALS AND METHODS

Chemicals. Azoxystrobin, cyprodinil, fludioxonil, mepanipyrim, pyrimethanil, and tetraconazole were obtained from the manufacturers. Triphenyl phosphate (99%) was used as the internal standard (i.s.) and was of analytical grade (Janssen, Geel, Belgium). Standard stock solutions (∼500 mg/L) were prepared in methanol. Working standard solutions were obtained by dilution with a hexane extract, containing the i.s. at 0.3 mg/kg, from culture media and wine without pesticide residues. Methanol and hexane were of HPLC grade (Carlo Erba, Milan, Italy). Ethanol was a pure reagent (Carlo Erba).

Culture Media. *(A) Yeasts.* Each insecticide was dissolved in ethanol (5 mL) and added to 1 L of YNBG broth, made up of 7 g/L yeast nitrogen base (YNB) and 180 g/L glucose (G) at pH 3.6. All media were sterilized by filtration through 0.22 μm membrane filters (Millipore, Molsheim, France).

(B) Bacteria. Each insecticide was dissolved in ethanol (5 mL) and added to 1 L of Vermentino wine containing 12% ethanol, 0.45 g/L volatile acidity, 6.2 g/L total acidity, and 4.40 g/L malic acid. The wine was sterilized by filtration through 0.22 μm membrane filters.

Inoculation and Fermentation. *(A) Yeasts.* The yeasts were *Saccharomyces cerevisiae* strain S and *Kloeckera apiculata* strain K from the collection of the Dipartimento di Scienze Ambientali Agrarie e Biotecnologie Agroalimentari, University of Sassari, Italy. Precultures were prepared with a substrate of 5% glucose and 0.7% YNB and agitated on a rotary shaker at 120 rpm for 48 h. The cells were then washed twice and suspended in 0.15 M NaCl. The amounts of the suspensions used as inocula were such to ensure 5×10^6 cells/mL in each of the culture media. For each strain and for each fungicide, after inoculation, the culture medium was apportioned into three 150-mL replications in 500-mL flasks. Two different controls were prepared, consisting respectively of a culture medium without inoculum (YNBG plus pesticide) to check the pesticide chemical degradation and an inoculated YNBG broth (no pesticide) to check fermentation. Each experiment was carried out in triplicate. All of the flasks were put to ferment in a thermostatically controlled chamber at 20 °C.

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Table 1. Effect of Pesticides on Fermentation Activity of *S. cerevisiae* and *K. apiculata* Yeasts

pesticide added	*S. cerevisiae*, after inoculation									*K. apiculata*, after inoculation								
	0 days			4 days			10 days			0 days			4 days			10 days		
(mg/L)	cell/mL	pH	CO_2[a]	cell/mL	pH	CO_2[a]	cell/mL	pH	CO_2[a]	cell/mL	pH	CO_2[a]	cell/mL	pH	CO_2*	cell/mL	pH	CO_2[a]
control	5.0×10^6	3.6	nd[b]	1×10^8	3.0	6.0	9×10^7	3.2	9.0	5.0×10^6	3.6	nd	6×10^7	3.3	1.0	5×10^7	3.4	2.5
Azoxystrobin																		
2.2	5.0×10^6	3.6	nd	7×10^7	3.6	7.0	8×10^7	3.6	9.5	5.0×10^6	3.6	nd	3×10^7	3.6	2.2	8×10^7	3.6	6.0
Cyprodinil																		
5.5	5.0×10^6	3.6	nd	6×10^7	3.6	8.0	9×10^7	3.6	9.3	5.0×10^6	3.6	nd	4×10^7	3.6	1.3	8×10^7	3.6	2.8
Fludioxonil																		
3.3	5.0×10^6	3.6	nd	6×10^7	3.0	6.7	9×10^7	3.2	9.5	5.0×10^6	3.6	nd	4×10^7	3.3	1.8	8×10^7	3.4	5.3
Mepanipyrim																		
3.50	5.0×10^6	3.6	nd	7×10^7	2.9	7.0	9×10^7	3.4	10.8	5.0×10^6	3.6	nd	4×10^7	3.3	2.5	8×10^7	3.4	7.0
Pyrimethanil																		
5.0	5.0×10^6	3.6	nd	8×10^7	3.0	8.0	9×10^7	3.4	11.5	5.0×10^6	3.6	nd	4×10^7	3.3	2.0	8×10^7	3.4	5.8
Tetraconazole																		
0.8	5.0×10^6	3.6	nd	7×10^7	3.0	7.0	8×10^7	3.4	10.5	5.0×10^6	3.6	nd	5×10^7	3.3	2.5	8×10^7	3.4	6.3

[a] Expressed as alcohol % (v/v). [b] nd, not detectable.

Table 2. Pesticide Residues (Milligrams per Kilogram ± SD) during Alcoholic Fermentation of *S. cerevisiae* (S) and *K. apiculata* (K) Yeasts

pesticide	sample	pesticide residues after			
		0 days	1 day	4 days	10 days
azoxystrobin	control	2.14 ± 0.29	2.20 ± 0.10	2.18 ± 0.07	2.23 ± 0.15
	S	2.14 ± 0.21	2.25 ± 0.23	2.18 ± 0.23	2.13 ± 0.15
	K	2.10 ± 0.21	2.19 ± 0.13	2.09 ± 0.21	2.33 ± 0.14
cyprodinil	control	5.34 ± 0.06	5.40 ± 0.04	5.46 ± 0.07	5.41 ± 0.15
	S	5.39 ± 0.12	5.41 ± 0.13	5.41 ± 0.13	5.31 ± 0.13
	K	5.47 ± 0.14	5.52 ± 0.16	5.38 ± 0.23	5.55 ± 0.22
fludioxonil	control	3.27 ± 0.28	3.24 ± 025	3.37 ± 0.24	3.07 ± 0.31
	S	3.31 ± 0.19	3.26 ± 0.29	3.22 ± 0.19	3.32 ± 0.25
	K	3.21 ± 0.10	3.21 ± 0.21	3. 09 ± 0.12	3.13 ± 0.15
mepanipyrim	control	3.42 ± 0.25	3.28 ± 0.23	3.12 ± 0.34	2.36 ± 0.23
	S	3.57 ± 0.13	3.32 ± 0.23	3.15 ± 0.32	2.48 ± 0.36
	K	3.29 ± 0.32	3.07 ± 0.08	2.99 ± 0.17	2.67 ± 0.18
pyrimethanil	control	4.92 ± 0.12	4.98 ± 0.17	5.19 ± 0.13	5.04 ± 0.18
	S	4.90 ± 0.21	3.84 ± 0.14	3.23 ± 0.16	3.02 ± 0.22
	K	4.89 ± 0.19	4.00 ± 0.12	4.17 ± 0.14	3.92 ±0.45
tetraconazole	control	0.73 ± 0.05	0.76 ± 0.02	0.73 ± 0.03	0.76 ± 0.02
	S	0.76 ± 0.05	0.76 ± 0.04	0.70 ± 0.06	0.74 ± 0.03
	K	0.74 ± 0.04	0.78 ± 0.05	0.78 ± 0.05	0.75 ± 0.05

(B) Bacteria. Two commercial lactic bacteria, *Leuconostoc oenos* strain L and *Lactobacillus plantarum* strain Plant, were used. Precultures were prepared in MRS (Oxoid, Hampshire, U.K.) broth for 5 days without agitation and inured to alcohol (increasing rates by 5, 7, and 10%). The amounts of suspensions used as inocula were such to ensure 5×10^9 cells/mL in wine. For each strain and for each fungicide, after inoculation, 150 mL of wine was apportioned in 500-mL flasks. All of the flasks were allowed to incubate in a thermostatically controlled chamber at 32 °C. Two different controls were prepared, consisting respectively of wine with inoculum to check the fermentative activity by acid malic degradation and wine with pesticide to check the pesticide stability. Each experiment was carried out in triplicate.

Samplings and Statistical Analysis. Four samplings were carried out after inoculation. Besides pesticide determination, the following analyses were made: pH, yeast cells per milliliter (microscopic count and culture count), and CO_2 production (indirect weighing). Data were processed by a statistical package for the analysis of variance.

Sample Preparation. For pesticide determination, each sample of alcoholic fermentation was accurately homogenized with a vibrating shaker (Velp Scientifica, Milano, Italy), and a 5-mL aliquot was vacuum filtered through regenerated cellulose membrane filters (∅ 25 mm, 0.45 μm) (Sartorius, Göttingen, Germany) to separate the yeasts from the fermentation liquid. The filter was washed with 1 mL of 10% ethanol. The filtrate and the wash solutions were analyzed separately.

Extraction Procedure. The following extraction procedure was carried out for filters (containing the yeasts), filtrates plus wash solutions, and 5 mL of wine. The sample was transferred into a 20-mL screw-capped tube; 5 mL of hexane containing the internal standard were added, and the tube was shaken in a rotary shaker for 30 min. After separation of the phases (by centrifugation, if necessary), the hexane layer was injected for GC analysis.

Pesticide Analysis. For the pesticide determination, previously described methods (Cabras et al., 1997c, 1998b) were used.

Apparatus and Chromatography. A gas chromatograph HRGC Mega 2 (Carlo Erba, Milano, Italy) equipped with a nitrogen−phosphorus detector (NPD-80), an AS 550 autosampler (Carlo Erba), and a split−splitless injector, connected to an HP 3396-A reporting integrator (Hewlett-Packard, Avondale, PA), were used. The capillary column was a Durabond fused silica (30 m × 0.25 mm i.d.) (J&W Scientific, Folsom, CA) DB-17 liquid phase (film thickness = 0.25 μm). The injector and detector were operated at 250 and 280 °C, respectively. The sample (2 μL) was injected in the splitless mode (60 s), and the oven temperature was programmed as follows: 110 °C for 1 min, raised to 280 °C (20 °C/min), and held for 10 min. Helium was the carrier and makeup gas at

Table 3. Malic Acid (Grams per Kilogram ± SD) during Malolactic Fermentation of *Le. oenos* (L) and *La. plantarum* (P) Lactic Bacteria

		pesticide residues after			
pesticide	sample	0 days	10 days	20 days	30 days
control	L	4.40	3.13 ± 0.04	2.37 ± 0.06	1.83 ± 0.04
	P	4.40	3.24 ± 0.04	2.51 ± 0.03	2.11 ± 0.01
azoxystrobin	L	4.40	3.75 ± 0.01	2.82 ± 0.04	2.10 ± 0.02
	P	4.40	3.70 ± 0.05	2.92 ± 0.03	2.30 ± 0.01
cyprodinil	L	4.40	3.71 ± 0.03	2.83 ± 0.07	2.09 ± 0.05
	P	4.40	3.67 ± 0.07	2.84 ± 0.05	2.17 ± 0.03
fludioxonil	L	4.40	3.71 ± 0.01	2.88 ± 0.03	2.17 ± 0.02
	P	4.40	3.62 ± 0.07	2.84 ± 0.05	2.24 ± 0.06
mepanipyrim	L	4.40	3.57 ± 0.01	2.53 ± 0.03	1.96 ± 0.05
	P	4.40	3.59 ± 0.04	2.81 ± 0.03	2.21 ± 0.01
pyrimethanil	L	4.40	3.67 ± 0.01	2.77 ± 0.02	2.07 ± 0.01
	P	4.40	3.71 ± 0.04	2.88 ± 0.04	2.18 ±0.02
tetraconazole	L	4.40	3.71 ± 0.06	2.78 ± 0.02	2.17 ± 0.01
	P	4.40	3.68 ± 0.08	2.86 ± 0.01	2.22 ± 0.02

Table 4. Pesticide Residues (Milligrams per Kilogram ± SD) during Malolactic Fermentation of *Le. oenos* (L) and *La. plantarum* (P) Lactic Bacteria

		pesticide residues after			
pesticide	sample	0 days	10 days	20 days	30 days
azoxystrobin	control	0.48 ± 0.05	0.46 ± 0.03	0.48 ± 0.04	0.44 ± 0.03
	L	0.48 ± 0.03	0.48 ± 0.04	0.44 ± 0.06	0.43 ± 0.03
	P	0.44 ± 0.01	0.43 ± 0.01	0.42 ± 0.02	0.44 ± 0.03
cyprodinil	control	1.18 ± 0.09	1.12 ± 0.02	1.20 ± 0.04	1.22 ± 0.01
	L	1.14 ± 0.05	1.22 ± 0.06	1.16 ± 0.05	1.18 ± 0.04
	P	1.12 ± 0.07	1.08 ± 0.04	1.09 ± 0.04	1.13 ± 0.07
fludioxonil	control	0.91± 0.03	0.90 ± 0.02	0.92 ± 0.02	0.90 ± 0.02
	L	0.93 ± 0.04	0.96 ± 0.02	0.95 ± 0.02	0.95 ± 0.02
	P	0.93 ± 0.03	0.93 ± 0.02	0.93 ± 0.02	0.95 ± 0.02
mepanipyrim	control	1.49 ± 0.11	1.52 ± 0.13	1.52 ± 0.12	1.52 ± 0.11
	L	1.77 ± 0.02	1.78 ± 0.08	1.87 ± 0.08	1.94 ± 0.15
	P	1.51 ± 0.14	1.49 ± 0.11	1.49 ± 0.12	1.50 ± 0.10
pyrimethanil	control	0.73 ± 0.02	0.76 ± 0.03	0.76 ± 0.02	0.75 ± 0.05
	L	0.69 ± 0.05	0.69 ± 0.07	0.69 ± 0.02	0.72 ± 0.05
	P	0.71 ± 0.04	0.65 ± 0.06	0.74 ± 0.04	0.72 ±0.01
tetraconazole	control	0.94 ± 0.07	0.93 ± 0.01	0.95 ± 0.12	0.92 ± 0.13
	L	0.96 ± 0.02	0.94 ± 0.04	0.98 ± 0.05	0.98 ± 0.01
	P	0.88 ± 0.06	0.90 ± 0.04	0.91 ± 0.03	0.90 ± 0.01

120 and 130 kPa, respectively. Calibration graphs for the active ingredients were constructed with the i.s. method by measuring peak heights versus concentrations. Good linearity was achieved in the 0.25−6.00 mg/kg range for all pesticides, with correlation coefficients between 0.9987 and 0.9995.

RESULTS AND DISCUSSION

Alcoholic Fermentation. To evaluate whether the presence of pesticides above the maximum residual limits (MRL) can negatively affect the fermentative process of wine, all experiments were carried out using a pesticide concentration higher than the MRL fixed in Italy for grapes (∼1.5 times) and found in field trials at harvest (∼10 times) (Cabras et al., 1997b, 1998a). Table 1 shows that the presence of pesticides, also in high concentrations, does not affect the alcoholic fermentation by *S. cerevisiae* and *K. apiculata*. Even though the number of cells in the control sample was higher than in the samples with pesticides during the first days of fermentation, the amount of alcohol produced was not negatively affected. This was due to the presence of a sufficient number of cells in all of the samples to allow a regular fermentative process. The presence of pesticides seems to stimulate a higher production of alcohol from yeast. This fact was especially observed in the case of *K. apiculata*, for which 2−3-fold increments of alcohol were observed with all pesticides except cyprodinil.

During the fermentative process pesticide residues were determined both on the liquid phase and on the yeasts. All of the pesticides were found only in the liquid phase, and the yeast did not affect the pesticide residue during the fermentative process (Table 2). Only pyrimethanil showed decreases of ∼20 and ∼40% during fermentation by *K. apiculata* and *S. cerevisiae*, respectively.

Malolactic Fermentation. The degradation of malic acid during malolactic fermentation followed a first-order kinetics, which was faster with *Le. oenos* ($t_{1/2}$ = 23.8 days; $r = -0.998$) than with *La. plantarum* ($t_{1/2}$ = 28.1 days; $r = -0.993$). During fermentation by *Le. oenos* the amount of malic acid was affected by the presence of pesticides, except mepanipyrim, with a lower degradation of ∼15%, whereas the difference observed during fermentation by *La. plantarum* was of ∼5%.

The amount of all pesticides was not affected significantly during malolactic fermentation.

Conclusion. The presence of the pesticides studied did not affect alcoholic fermentation, not even with higher levels than those normally found in grapes in field experiments. On the contrary, it stimulated yeast to produce more alcohol, especially with *K. apiculata*. The fermentative process did not affect the amount of pesticides either by degradation or by adsorption, except pyrimethanil. During malolactic fermentation malic acid underwent a lower degradation (∼15%) in the presence of all pesticides, except mepanipyrim, by *Le. oenos*, whereas by *La. plantarum* the decrease was lower, ∼5%. The bacteria studied did not show any degradative effect on pesticides during malolactic fermentation.

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