CHAPTER 2

Pesticides' Influence on Wine Fermentation

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Contents

Abstract

Wine quality strongly depends on the grape quality. To obtain high-quality wines, it is necessary to process healthy grapes at the correct ripeness stage and for this reason the farmer has to be especially careful in the prevention of parasite attacks on the grapevine.

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The most common fungal diseases affecting grape quality are downy and powdery mildew (*Plasmopara viticola* and *Uncinula necator*), and gray mold (*Botrytis cinerea*). On the other hand, the most dangerous insects are the grape moth (*Lobesia botrana*), vine mealybug (*Planococcus ficus*), and the citrus mealybug (*Planococcus citri*).

Farmers fight grape diseases and insects applying pesticides that can be found at harvest time on grapes. The persistence of pesticides depends on the chemical characteristic of the active ingredients as well as on photodegradation, thermodegradation, codistillation, and enzymatic degradation. The pesticide residues on grapes can be transferred to the must and this can influence the selection and development of yeast strains. Moreover, yeasts can also influence the levels of the pesticides in the wine by reducing or adsorbing them on lees. During the fermentative process, yeasts can cause the disappearance of pesticide residues by degradation or absorption at the end of the fermentation when yeasts are deposited as lees.

In this chapter, we reviewed the effect of commonly used herbicides, insecticides, and fungicides on yeasts. We also studied the effect of alcoholic and malolactic fermentation on pesticide residues.

I. INTRODUCTION

The grapevine requires particular climatic conditions and, for this reason, grapes can be cultivated only in the temperate zones of the two hemispheres. These zones lie between 50° and 30° of north latitude and from 30° to 40° of south latitude (Fig. 2.1).

The highest concentration of grapevine cultivation is located in the Mediterranean basin where France, Italy, and Spain are the largest producing nations. In the North America, California is the U.S. state with the highest production of wine, while Chile and Argentina are the most important grape-producing nations on the South American continent. Recently, Australia and New Zealand have emerged as significant grape-producing nations. Worldwide, the grapevine is cultivated on eight million hectares, and the wine production reaches about 260 million hectoliters. The EU possesses 64% of the total grapevine cultivation, which corresponds to the ¾ of the worldwide wine production. France and Italy compete for the top rank in wine production; they have constantly battled for first and second place in any given vintage-year.

Nevertheless, *per capita* wine consumption is progressively diminishing, most importantly in those top producing countries such as France,

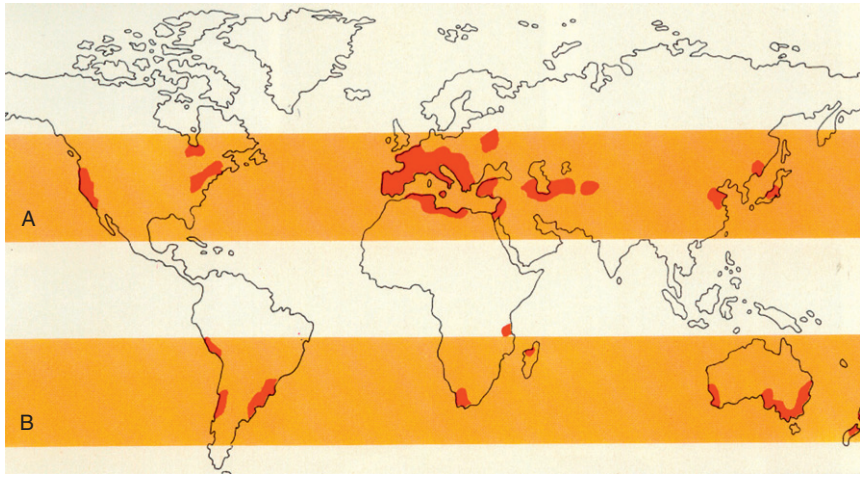

FIGURE 2.1 Worldwide distribution of grape cultivation shown in dark shade.

Spain, and Italy. For example, the Italian average *per capita* wine consumption decreased from 119.6 to 99.8 L from the beginning of the century through 1960. After a progressive rise in wine consumption reaching the maximum of 116 L *per capita* in 1968; a constant reduction has been observed from 94.6 L in 1978, to 90.6 L in 1980, to 61.5 in 1990, to 57.6 L in 1995, to 57.2 L in 1996, and to 46.5 in 2005. In the new wine-producing countries, such as New Zealand, individual consumption continues to grow quickly (20.3 L *per capita* in 2005, which is a two-fold increase in 6 years). Countries with the highest *per capita* wine consumption are reported in Table 2.1.

II. GRAPEVINE PATHOGENS

There is no question that the wine quality strongly depends on the grape quality. To obtain high-quality wines, it is necessary to use healthy grapes at the correct ripeness stage and for this reason the farmer has to be especially careful in the prevention of parasite attacks on the grapevine. Many of the grapevine parasites are of animal origin (insects and mites) or from vegetal origin (critograme or parasitic fungi). The most common fungal diseases are downy and powdery mildew (*Plasmopara viticola* and *Uncinula necator*), and gray mold (*Botrytis cinerea*). On the other hand, the most dangerous insects are the grape moth (*Lobesia botrana*), vine mealybug (*Planococcus ficus*), and the citrus mealybug (*Planococcus citri*).

TABLE 2.1 World *per capita* wine consumption

Countries	*Per capita* wine consumption (L)
France	55.4
Luxembourg	54.6
Portugal	46.7
Italy	46.5
Slovenia	44.7
Croatia	40.8
Switzerland	39.3
Hungary	34.7
Greece	32.2
Spain	31.8
Austria	29.3
Denmark	28.7

Year 2005, source O.I.V.

A. Downy mildew (*P. viticola*)

The downy mildew was introduced in France from North America in 1878. It moved into Italy the following year and subsequently into the other countries of the Mediterranean basin. Furthermore, it was introduced into Australia in 1919 and into New Zealand in 1926. Today downy mildew is present in all vine cultivation areas.

The downy mildew is the most prevalent form of mildew and is usually spread by rainfall. It attacks leaves, shoots, and berries and can quickly defoliate the vine leading to loss of the entire crop. Optimum conditions for primary infection take place at 10:10:10, which corresponds to at least 10 mm of rain at a temperature 10 °C or more, over 10 h. The fungus survives in the form of spores for 3–5 years in old, infected leaf material that is remaining in the soil and, with rain, is splashed onto the foliage. If the spores remain wet long enough, the disease begins to develop. This shows up as ''oil spots'' on leaves. Spores form under the oil spot and show up as a ''white down.'' If conditions are right, secondary infection occurs from these spores and the spread of the disease becomes quite rapid.

Downy mildew can be controlled by the spray application of various chemicals either as preinfection or postinfection treatments. There are two groups of spray chemicals, those with single site activity which act on only one site within the fungus organism or those with multisite activity, which act on more than one site within the fungus. The most used multisite chemicals for the preventive control of downy mildew are

copper salts such as copper oxychloride. In the past several years, the overuse of chemicals has lead to small mutational changes within the fungus, which in turn can lead to the fungus being resistant.

At present, many of the nonsystemic active ingredients against downy mildew such as metiram, mancozeb, folpet, tolylfluanide are commonly used. On the other hand, the systemic fungicides in current use are cymoxanyl, dimethomorf, famoxadone, fenamidone, zoxamide, metalaxil-m, iprovalicarb, and strobirulines (azoxystrobin and pyraclostrobin).

B. Powdery mildew (*U. necator*)

The powdery mildew is a pathogen that was brought into England from North America in 1845. Subsequently, powdery mildew was introduced into France in 1847, Belgium in 1848, and finally Italy in 1849. By 1853, it was discovered, in France, that vine treatments with sulfur were able to control this pathogen.

Powdery mildew attacks leaves, shoots, and bunches. It is evidenced by an ash gray to white powdery growth on both the upper and lower surfaces of the leaves. Moreover, the disease attacks the bunches with the same ash gray/white powder showing up on the berries and stalks. Other than crop losses, the most negative aspect is that the disease causes off flavors in wine production.

Powdery mildew spores hide in the buds of dormant vines. Mild cloudy weather and low light in the canopy encourage development of this disease.

There are no approved fungicides for postinfection treatments that make the application of a protective spray from budburst necessary. There are multiple chemicals from both the singlesite and multisite groups. In Italy, the relatively safe and multisite active wettable sulfur is utilized. Other than sulfur, many active ingredients such as dinocap, fungicides, QoI-STAR derived from strobilurins (azoxystrobin, kresoxy-methyl, trifloxystrobin), quinoxyfen and IBS (inhibitors of sterol biosynthesis) such as fenarimol, triadimenol, penconazole, myclobutanil, fenbuconazole, hexaconazole, fusilazole, tetraconazole, and tebuconazole are used. Other active ingredients used are proquinazid and spiroxamine.

C. Gray mold (*B. cinerea*)

Gray mold is a common bunch rot in regions with warm, wet conditions. In addition to the fruit, it can also attack shoots and leaves. It causes large crop losses while infected grapes can cause off flavors in the wine. It should be mentioned here that not all *botrytis* infections are unfavorable. Under specific conditions, the fungus takes hold and dehydrates the bunches increasing the sugar content without causing rot. This enables very sweet dessert wines with their traditional marmalade favor caused

by the action of fungal enzymes (e.g., Sauterne in France or in Australia). The disease in this case is known as noble rot. The disease hides in decaying plant debris such as dead canes and mummified fruit. Spores are spread by wind and find a place in the developing bunch flowers. If the ''closed'' bunch coincides with wet weather and high humidity, the disease spreads rapidly.

There are virtually no curative sprays, and it is essential that a protective spray is applied at very definite times of bunch development. Applications are commonly at 80% capfall (toward the end of flowering) and again just before bunch closure (just before the berries have stopped growing and become ''squished'' together in the bunch). Chlorothalonil is commonly used for this purpose. This chemical is also a protectant against downy mildew so it can replace the copper. Pesticides used to control botrytis are the following: dicarboxymides (iprodione, procymidone, and vinclozonil), new generation products such as pyrimethanil, mepanypirim, fenhexamid, ciprodinil + fludioxonil, and fluazinam.

D. Grape moth (*L. botrana*)

The life cycle of *L. botrana* can allow 3–4 generations depending on geographical and environmental variability and whether the summer has been hot. The moths first appear at the end of April when the vine has 3 or 4 leaves and they emerge at intervals and the flights spread over 2–3 weeks. The caterpillar finishes its development at the time of flowering and then it pupates. The second flight takes place toward the end of June and into July; then the caterpillars pupate again and the third flight occurs between mid-August and the end of September. The caterpillars gnaw the almost ripe fruits and various molds, in particular *Botrytis*, develop very rapidly on the wounds; the attacked fruits turn brown at the place of attack and begin to rot. The presence of larvae and rotten fruits lowers the quality of the crop; molds render wine making difficult and may require the crop to be harvested prematurely. The following pesticides are commonly used to control the grape moth: pyrethroids (cypermethrin and deltamethrin), organophosphorus (chlorpyrifos, chlorpyrifos-methyl), nicotinoids (imidacloprid), oxadiazine insecticides (indoxacarb), chitin synthesis inhibitor insecticides (flufenoxuron, lufenuron), and moulting hormone agonists (tebufenozide).

E. Vine and the citrus mealybugs (*P. ficus* and *P. citri*)

The two insects, morphologically similar, are the vine mealybug (*P. ficus*), and the citrus mealybug (*P. citri*). They are currently the most economically important pseudococcids in vineyards in Italy. All life stages of vine mealybug are found throughout the vine, including on the roots, under

the bark on the trunk and cordons, on canes, and leaves. There is no overwintering stage, rather all life stages can be found throughout the year. There are usually 3–7 generations per year. During the winter months, eggs, nymphs, and adults can be found under the bark, within developing buds, and on the roots as well. As temperatures warm in the spring, the density of vine mealybug increases, and the mealybugs move out to the cordons and aerial parts of the vine. Vine mealybug can be found on all parts of the vine including leaves and clusters by late spring and summer. Shortly after harvest, the density of vine mealybug declines. This generalized biology fits most vine mealybug populations; however, it varies slightly with location and cultivar.

At high densities, the vine mealybugs can reduce plant vigor by removing large amounts of sap, which carries the nutrients to the grape roots and growing tissues including the grape bunches. The vine mealybugs excrete large amounts of fluids that have high concentrations of sugars. This ''honeydew'' can foul the grapevine with a layer of sticky sap as it dries. In addition, a fungus called ''sooty mold'' grows on the honeydew. This black fungus covers the grape leaves interfering with photosynthesis and fouling the grape bunches. The vine mealybug is known to transmit leaf roll virus in grapes. This same behavior is exhibited in *P. citri*.

Generally, the chemical control is done in the spring time to coincide with the emerging of nymphs from winter sites using mineral oil or calcium polysulfur. Chlorpyrifos, chlorpyrifos-methyl, imidacloprid, methomyl, buprofezin, and dimethoate can be used as alternatives.

III. PESTICIDES

Before entering the market, pesticides need to be registered. Starting in 2008, pesticide registration has been done by the EU and not by individual countries. The registration process for each pesticide set requires the authorized culture, the dose, preharvest interval, and the maximum residue limit (MRL). The legal limit of the residue does not coincide with the toxicological limit and for this reason still if the legal limit is exceeded it will not pose a serious risk to human health. The legal limit is determined from toxicological data establishing a lack of risk to human health (NOEL = no observed effect level) commonly corrected by a safety factor of 100. Field residues of pesticides are affected by the environmental conditions (temperature, wind, rain, solar irradiance, etc.). Field residues, if below the corrected acceptable daily intake, are used to set the legal limit of the pesticide residue. Residues limits can vary between countries because of the different climatic conditions, leading to EU trade difficulties.

Currently the EU is working for the harmonization of the MRLs of pesticides. In Italy, pesticides currently registered for use on grapes are listed in Table 2.2. Italy is one of the few countries with legal limits also set on wine (Table 2.3**)**. In other countries, where there is a lack of a legal limit for processed foods, the amount of the raw food corresponding to processed food unit (e.g., 1.5 kg of grapes for 1 L of wine), and the incidence of technological process should be taken into account. Since each active ingredient has its particular behavior, residue changes during the transformation process should be determined. In the absence of these data, the unique and safe reference is the MRL of the primary food.

Different levels of pesticides can be found at harvest on grapes depending on the chemical characteristic of the active ingredients. Moreover, the persistence of pesticides can depend on photodegradation, thermodegradation, codistillation, and enzymatic degradation.

The pesticide residues on grapes can be transferred to the must and this can influence the selection and development of yeast strains. Moreover, yeasts can also influence the levels of the pesticides in the wine by reducing or adsorbing them on lees (Cabras *et al.*, 1987).

IV. FERMENTATION PROCESS

In the fermentative process, the first step is due to yeasts which transform sugars to alcohol (alcoholic fermentation). This is followed by a second fermentation step (malolactic fermentation), which corresponds to the transformation of L-malic acid to L-lactic acid.

A. Alcoholic fermentation

In winemaking, the fermentative process may take place due to ambient yeasts that are naturally present in wine cellars, vineyards and on the grapes themselves (sometimes known as a grape's ''bloom''). Otherwise, it can be conducted using cultured yeast which are specifically isolated and inoculated for use in winemaking. Yeasts responsible for alcoholic fermentation belong to the genus *Saccharomyces* spp. However, other yeasts, especially non-*Saccharomyces* yeasts are present in the initial stages of the fermentation process and may have an influence on the final organoleptic properties of the wine (Pretorius *et al.*, 1999). These genera include *Candida, Klöckera/Hanseniaspora, Pichia,* and *Zygosaccharomyces*. These yeasts grow to about 10^6, 10^7 cfu/mL but, by midfermentation begin to decline and die off. At this time, *Saccharomyces cerevisiae* becomes predominant (10^7, 10^8 cfu/mL) and continues the fermentation until its completion. Evidence exists that non-*Saccharomyces* yeasts may influence the unique oenological characteristics of each wine-producing zone, while

TABLE 2.2 Pesticides registered on grapes in Italy

Pesticide	MRL (mg/kg)	Pesticide	MRL (mg/kg)	Pesticide	MRL (mg/kg)
Abamectin	0.01	Esfenvalerate	0.1	Methoxyfenozide	1
Acrinathrin	0.1	Ethephon	0.1	Metiram	2
Alcalines solphites	10	Etofenprox	0.05	Myclobutanil	1
Alphamethrin	0.3	Etoxazole	1	Oxadiazon	0.05
Azadirachtin	0.5	Famoxadone	0.02	Oxyfluorfen	0.05
Azinphos-methyl	1	Fenamidone	2	Paraquat	0.05
Azociclotin	0.3	Fenamiphos	0.5	Penconazole	0.2
Azoxystrobin	2	Fenarimol	0.02	Phosalone	1
Benalaxyl	0.2	Fenazaquin	0.3	Phosetyl-al	2
Benfuracarb	0.05	Fenbuconazole	0.2	Piperonyl butoxide	3
Bifenthrin	0.2	Fenbutatin oxide	0.2	Pirimicarb	0.2
Bifentrin	0.2	Fenhexamid	2	Pirimiphos-methyl	2
Bromopropylate	2	Fenoxycarb	0.5	Procymidone	5
Bromuconazole	0.5	Fenpropidin	0.2	Propargite	2
Buprofezin	1	Fenpropimorph	2	Propiconazole	0.5
Calcium polysulfide	50	Fenpyroximate	0.05	Propineb	2
Captan	10	Flazasulfuron	0.3	Propyzamide	0.02
Carbaryl	3	Fluazifop-*p*-butyl	0.01	Pyraclostrobin	2
Carbendazim	2	Fluazinam	0.1	Pyrethrins	1
Chloropicrin	0.05	Fludioxonil	1	Pyridaben	0.1
Chlorothalonil	3	Fludioxonil	2	Pyrimethanil	3
Chlorpropham	0.05	Flufenoxuron	2	Quinoxyfen	0.5

(*continued*)

TABLE 2.2 (*continued*)

Pesticide	MRL (mg/kg)	Pesticide	MRL (mg/kg)	Pesticide	MRL (mg/kg)
Chlorpyrifos	0.5	Flusilazole	0.1	Rotenone	0.05
Chlorpyrifos-methyl	0.2	Fluvalinate	0.01	Spinosad	0.2
Clofentezine	1	Folpet	0.5	Spiroxamine	1
Cyanamide	0.05	Glufosinate ammonium	10	Sulfur	50
Cyazofamid	1	Glyphosate	0.1	Tebuconazole	1
Cycloxidim	0.1	Glyphosate trimesium	0.1	Tebufenozide	0.5
Cyfluthrin	0.3	Hexaconazole	0.1	Tebufenpyrad	0.3
Cyhexatin	0.3	Hexythiazox	0.5	Teflubenzuron	1
Cymoxanil	0.1	Indoxacarb	0.5	Tetraconazole	0.5
Cypermethrin	0.5	Iprodione	10	Thiamethoxam	0.5
Cyproconazole	0.2	Iprovalicarb	2	Thiodicarb	1
Cyprodinil	5	Kresoxim-methyl	1	Thiram	3.8
Deltamethrin	0.1	Lambda cyalothrin	0.2	Tiophanate-methyl	2
Diazinon	0.02	Lufenuron	0.5	Tolylfluanid	5
Dichlobenil	0.1	Mancozeb	2	Triadimenol	2
Dichlorvos	0.1	Maneb	2	Trichlorfon	0.5
Dicofol	2	Mcpa	0.1	Trifloxystrobin	3
Diethofencarb	1	Mecoprop	0.1	Trifluralin	0.05
Dimethomorph	0.5	Mepanipyrim	3	Vinclozolin	5
Diquat	0.05	Metalaxil-m	1	White mineral oil	0
Dithianon	0.6	Metam-sodium	2	Zeta cypermethrin	0.5
Diuron	0.05	Methidathion	0.5	Ziram	2
Dodine	0.2	Methiocarb	0.05	Zoxamide	5
Endosulfan	0.5	Methomyl	1		

TABLE 2.3 Maximum residue limits (mg/L) in grape and wine

Pesticide	Grape	Wine
Azoxystrobin	2	0.5
Bromuconazole	0.5	0.2
Buprofezin	1	0.5
Cyazofamid	1	0.05
Cyproconazole	0.2	0.02
Cyprodinil	5	0.5
Dazomet	–	0.02
Diethofencarb	1	0.3
Etofenprox	1	0.1
Etoxazole	0.02	0.01
Fenamidone	0.5	0.5
Fenazaquin	0.2	0.01
Fenhexamid	5	1.5
Fenpropidin	2	0.5
Flazasulfuron	0.01	0.01
Fluazinam	1	0.02
Fludioxonil	2	0.5
Hexaconazole	0.1	0.01
Indoxacarb	0.5	0.02
Iprodione	10	2
Iprovalicarb	2	1
Mepanipyrim	3	1
Metalaxil-m	1	0.2
Metam-sodium	2	0.2
Methoxyfenozide	1	0.05
Myclobutanil	1	0.1
Procymidone	5	0.5
Pyrimethanil	3	2
Quinoxyfen	0.5	0.01
Spinosad	0.2	0.01
Spiroxamine	1	0.5
Tebuconazole	1	0.5
Tebufenozide	0.5	0.1
Tebufenpirad	0.3	0.1
Teflubenzuron	1	0.01
Thiamethoxam	0.5	0.5
Trifloxystrobin	3	0.3
Ziram	2	0.2
Zoxamide	5	0.5

the presence of pesticides can affect metabolic activity. Some wine producers, particularly in Europe, advocate use of ambient yeast as a characteristic of the region's *terroir*. On the other hand, many winemakers prefer to control the fermentation using a predictable cultured yeast. The cultured yeasts most commonly used in winemaking belong to the species *S. cerevisiae*. Within this species are several hundred different strains of yeast that can be used during fermentation to affect the heat or vigor of the process and enhance or suppress certain flavor characteristics of the varietal. The use of different strains of yeasts is a major contributor to the diversity of wine, even those produced using the same grape variety. The addition of cultured yeast normally occurs with the yeast beginning in a dried or ''inactive'' state and is reactivated in warm water or diluted grape juice prior to being added to the must. To thrive and be active in fermentation, the yeast needs to have access to a continuous supply of carbon, nitrogen, sulfur, and phosphorus as well as access to various vitamins and minerals. These components are naturally present in the grape must but their amount may be adjusted by adding nutrient packets to the wine, in order to foster a more encouraging environment for the yeast. Oxygen is needed as well but care needs to be taken in reducing the risk of oxidation and the lack of alcohol production from oxygenated yeast by keeping the exposure to oxygen at a minimum.

Among the oenological yeasts added during alcoholic fermentation, some strains of *S. cerevisiae* can use organic compounds to produce hydrogen sulfide and sulfites or the intermediate product of methionine (Zambonelli, 1988). These strains can use other sulfur-containing molecules, such as several of the pesticides employed in viticulture.

At the end of the fermentation process, the yeast exhausts its life cycle and falls to the bottom of the fermentation tank as sediment known as lees.

B. Pesticide effect on yeasts

During the fermentative process, yeasts can cause the disappearance of pesticide residues by degradation or absorption at the end of the fermentation when yeasts are deposited as lees.

Consequently, it is important to identify the different microbial groups present on the surface of the grapes. The surface of healthy grapes has a predominant microflora of *Metschnikowia*, *Hanseniaspora, Candida, Pichia, Rhodotorula*, and some *Saccharomyces* and *Zygosaccharomyces* species, while damaged grapes have increased populations of strains of yeasts that contribute to alcoholic fermentation. Generally, very few yeasts (10–10^3 cfu/g) are detected on immature grape berries, but they increase to populations of 10^4–10^6 cfu/g as the grape matures to harvest (Fleet, 2003).

Ethylenebisdithiocarbamates (EBDCs) were the first fungicides used in the field. Several authors reported that EBDCs do not affect the fermentative activity when present in such low concentration at vintage time (Cordier, 1954; Minarik and Regala, 1975). On the contrary, Schopfer (1978) reported that maneb concentrations higher than 10 mg/L inhibited yeast action, whereas metiram produced no effect in concentrations up to 100 mg/L. Conner (1983) studied the effect on yeasts of some fungicides, insecticides, and herbicides showing that insecticides such us methiocarb, rotenone and fungicides such as benomyl, copper oxychloride, iprodione, and vinclozonil were not toxic to yeast strains. On the other hand, the herbicide diuron was slightly toxic, while the insecticide dicofol was particularly toxic. Fort *et al.* (1999) observed that 5 mg/L of copper did not have a significant effect on the living yeast populations. Guerra *et al.* (1999) studied the effect on the populations of indigenous yeasts strains for two groups of pesticides. The first group of fungicides was comprised of copper salts, sulfur and myclobutanil, fenitrothion, metalaxil-m, penconazole, and vinclozonil. The second group was comprised of: copper sulfate, sulfur, and copper oxychloride. Within the second group, the authors isolated *Saccharomyces* species, while no *S. cerevisiae* could be isolated from the first group.

Batusic *et al.* (1999) described the effect of copper hydroxide and sulfur on different types of the species *S. cerevisiae* and *Saccharomyces bayanus*. The addition of these two products rapidly decreased the fermentation activity of *S. bayanus*, while it showed a lower intensity of fermentation with *S. cerevisiae*. Girond *et al.* (1989) showed that mancozeb, folpet, and myclobutanil were toxic to 284 different yeasts isolated from musts and grapes obtained from four French vineyards during the 1986 and 1987 vintage. Sapis-Domercq (1980) verified the influence of metalaxyl, belonging to acylalanines, on several of the yeasts (*S. cerevisiae*, *S. bayanus*, and *balii*; *Hanseniaspora uvarum*, *Candida mycoderma*), and found no effect on fermentation activity. Moreover, the author observed that dicarboximidic fungicides (iprodione, procymidone, and vinclozonil) did not show any effect on the yeasts. Dubernet *et al.* (1990) showed that myclobutanil and hexaconazole had a high toxicity toward yeasts. Folpet and captan, belonging to the class of phthalimides, were the first two chemicals used against *B. cinerea* in the early 1950s. In 1953, Peynaud and Lafourcade (1953) reported that captan had an antiseptic effect on *Saccharomyces*. Ehrenhardt and Jacob (1968) and Minarik and Regala (1975) both confirmed the fermentation inhibitory action of thiophthalimides. Gaia *et al.* (1978), after a three-year study on the effect of phthalimides on fermentation microbiology, reported the following findings: (a) all of these fungicides, particularly folpet delay fermentation; (b) even at high concentrations of 0.1 ppm they inhibit yeast cell development and reproduction; (c) they affect both quantity and quality of the spontaneous yeast

microflora in the grape and must, which reduces the fermentation by *S. cerevisiae*, while increasing the fermentation by *Candida.* Folpet degraded in the must. The presence of folpet in grapes inhibited the alcoholic fermentation of *S. cerevisiae* and *Kloeckera apiculata*. On the contrary, phthalimide had no negative effect on the alcoholic fermentation (Cabras *et al*., 1997a). Experiments with carbendazim residues, belonging to the benzimidazole class, up to 5 ppm (Bolay *et al*., 1972; Lemperle *et al*., 1970, 1971); and at 10 ppm (Gnaegi and Dufour, 1972) showed that alcoholic fermentation proceeded normally even though the residues were higher than the MRL. Thiophanate methyl, belonging to the thiophanate class, caused an appreciable delay in alcoholic fermentation (Gaia *et al*., 1978). Many authors reported that dicarboxymides (iprodione, procymidone, and vinclozonil) do not affect alcoholic fermentation, even if present in elevated concentrations in the must (Bolay *et al*., 1976; Faure *et al*., 1976; Lemperle *et al*., 1982; Sapis-Domercq *et al*., 1977, 1978; Schopfer, 1978). Fenarimol and cyprodinil, belonging to the class of pyrimidine fungicides, showed different behavior concerning their effects on fermentation. In specific, fenarimol delayed fermentation (Zironi *et al*., 1991) while cyprodinil, even when tested at high concentration levels, did not affect the alcoholic fermentation by *S. cerevisiae* and *Hanseniaspora/K. apiculata* (Cabras *et al*., 1999). Azoxystrobin, fludioxonil, mepanipyrim, pyrimethanil, and tetraconazole did not affect the alcoholic fermentation of *S. cerevisiae*. On the contrary, their presence stimulated the yeasts, and especially, *Hanseniaspora/K. apiculata*, to produce more alcohol (Cabras *et al*., 1999). Using *S. cerevisiae* as the yeast starter for alcoholic fermentation, no effects were observed in presence of azoxystrobin, chlorpyrifos-methyl, cyprodinil, fluazinam, fludioxonil, mepanipyrim, methidathion, pyrimethanil, and tetraconazole (Cabras *et al*., 1995a, 1997b, 1998). Calhelha *et al.* (2006) investigated the effects of benomyl, iprodione, procimidone, and vinclozolin on *Saccharomyces* and non-*Saccharomyces* yeasts present in the fermentation process. Benomyl had a negative effect on yeast growth, with *Zygosaccharomyces rouxii* and *S. cerevisiae* being the most resistant yeasts, while *Rhodotorula glutinis* was the most susceptible.

S. cerevisiae can produce H_2S and SO_2 in the presence of sulfur-containing insecticides such as chlorpyrifos-methyl, fenitrothion, and methidathion (Cabras *et al*., 1995b; Eschenbruch, 1974). These insecticides did not affect fermentative activity. Quinoxyfen, belonging to the family of quinolines, showed no effect on the alcoholic fermentation using three strains of yeasts (Cabras *et al*., 2000; Lopez *et al*., 2004).

Some wines such us Sherry (Spain), Vernaccia (Italy), and Jura yellow (France) are developed under oxidative conditions, utilizing yeasts known as Flor-yeast. These yeasts belong to the *S. cerevisiae* var. *prostoserdovii* which aid in the development of certain specific sensory characteristics (Fatichenti *et al*., 1983a,b). Studies were conducted with flor-yeast

(*S. cerevisiae* var. *prostoserdivii*) showing that film growth was not affected by carbendazim tested at the concentrations higher than 1.5 mg/L, while film growth was not complete with benalaxyl tested at concentrations higher than 6 mg/L, and was completely inhibited by triadimefon tested at concentrations higher than 30 mg/L (Farris *et al.*, 1989).

The majority of those experiments were done by using inoculated fermentations, but there remained a lack of results for the indigenous yeast flora in grapes in fermenting must. Čuš and Raspor (2008) studied the spontaneous wine fermentation with *S. cerevisiae* and *H. uvarum* with Pyrimethanil in the must. This fungicide had an effect on the course and successful conclusion of spontaneous wine fermentation. The initial *S. cerevisiae* concentration was significantly lower, while the *H. uvarum* concentration was higher in the must treated with Pyrimethanil.

C. Effect of alcoholic fermentation on pesticide residues

Lemperle *et al.* (1975) reported that most residues from EBCDs where adsorbed by scums and were not detectable in the wine. *S. cerevisiae*, a producer of H_2S and SO_2 (Cabras *et al.*, 1995b), adsorbed and degraded some sulfur-containing insecticides (Table 2.4). Chlorpyrifos methyl residues were partitioned with 65% of the initial amount in the liquid phase, 19% adsorbed by the yeasts, and about 15% of the active ingredient degraded. At the end of the fermentation, the fate of the residues was 26% in the liquid phase, 17% adsorbed by the yeasts, and about 43% of the active ingredient degraded. Fenitrothion, after inoculation, was distributed with about 90% in the liquid phase while 10% was adsorbed by the yeasts. At the end of the fermentation, this insecticide was distributed with approximately one-half and almost one-fourth of the initial value in the liquid and in the yeasts, respectively. No significant difference between the activities of the two yeast strains was observed. Methidathion was not adsorbed and did not undergo any degradation by the yeasts. The degraded pesticides belong to the thiophosphates (chlorpyrifos-methyl, and fenitrothion), while the dithiophosphates (methidathion) showed higher stability. The action of *S. cerevisiae* upon the insecticides carbaryl, deltamethrin, and vinclozonil, during aerobic fermentation was investigated (Cabras *et al.*, 1988). As reported in Table 2.4, yeasts initially adsorbed about 30% of carbaryl, while 50% was present in the liquid phase. Almost 20% of the initial amount of carbaryl was degraded. At the end of the fermentation, 35% was distributed in the liquid and about the same in the yeasts, while the remaining 30% of carbaryl was degraded. Deltamethrin was not present in fermentation liquid but was adsorbed by the yeasts with 59% present initially compared to 35% at the end of the fermentation. This fact showed that, during the fermentation, an additional 25% of the insecticide was degraded.

TABLE 2.4 Pesticides residues in the control, culture liquid and yeasts during fermentation by two strains

		Pesticide residues (mg/L)	
Pesticide	Sample	0 days	7 days
Chlorpyrifos methyl	Control	0.90	0.65
X	Liquid	0.59	0.17
	Yeast	0.17	0.11
Y	Liquid	0.51	0.16
	Yeast	0.19	0.12
Fenitrothion	Control	0.89	0.80
X	Liquid	0.85	0.85
	Yeast	ND	ND
Y	Liquid	0.85	0.86
	Yeast	ND	ND
Methidathion	Control	0.99	0.77
X	Liquid	0.91	0.70
	Yeast	ND	ND
Y	Liquid	0.95	0.68
	Yeast	ND	ND
Carbaryl	Control	2.91	3.00
	Liquid	1.33	1.06
	Yeast	0.91	1.08
Deltamethrin	Control	1.21	0.66
	Liquid	ND	ND
	Yeast	0.71	0.23
Vinclozonil	Control	1.90	1.71
	Liquid	ND	ND
	Yeast	1.75	1.95

X = *Saccharomyces cerevisiae* producer of H_2S; Y = *Saccharomyces cerevisiae* producer of SO_2.

Vinclozonil was not present in the fermentation liquid and was completely adsorbed by the yeasts at both the beginning and at the end of the fermentation. No evidence of degradative action by the yeasts was observed.

V. MALOLACTIC FERMENTATION

Malolactic fermentation (MLF) is an important secondary fermentation that occurs in many wines generally about 2–3 weeks after completion of the alcoholic fermentation. Lactic acid bacteria, principally *Oenococcus oeni* (formerly *Leuconostoc oenos*) are responsible for this fermentation.

This bacteria is naturally present in the wine or commercial strains maybe inoculated. Growth of the *O. oeni* during the fermentation functions to decrease wine acidity by transforming L-malic acid into L-lactic acid. *O. oeni* can also enhance wine flavor and complexity through the production of additional metabolites (Calhelha *et al.*, 2006). Grapes, especially if damaged, are a primary source of bacteria in the vinery environment (Wibowo *et al.*, 1985), while healthy grapes have low populations of bacteria ($<10^3$–10^4 cfu/mL). Many factors affect the growth of *O. oeni* in wines and the conduct of the MLF. Among these, pesticide residues can influence the growth of these microorganisms.

A. Pesticide effect on lactic bacteria

Certain fungicide and pesticide residues, especially the former, may have a detrimental effect on the functioning of malolactic bacteria. Most effective, in a negative sense, are residues of the systemic compounds often used in humid years to control the *botrytis* spp. Careful precautions should be taken in years with high incidence of *botrytis* contamination. Wine producers must be familiar with the spraying programs and products used, and they must adhere to the prescribed withholding periods required for the various antifungal products used. Damaged grapes have increased populations of lactic bacteria on the skin and surface grapes (Fleet, 2003). Grape juices produced from healthy, mature grapes have low populations (103–104 cfu/mL) of bacteria. These bacteria generally show little growth and die off to nondetectable levels (Fleet, 2001).

Radler and Schoning (1974) found that mancozeb markedly inhibited the activity of lactic bacteria. Sapis-Domercq (1980) verified the influence of metalaxyl on several bacteria (*Lactobacillus hilgardii*; *Leuconostoc fragile*), but found no effect on MLF. At the same time, lactic bacteria were not altered by dicarboximidic fungicides (iprodione, procymidone, and vinclozonil) even at high concentrations. The influence of six fungicides (azoxystrobin, cyprodinil, fludioxonil, mapanyperim, pyrimethanil, and tetraconazole) on two lactic bacteria (*L. oenos* and *Lactobacillus plantarum*) was studied (Cabras *et al.*, 1999). During MLF by *L. oenos*, malic acid decreased slightly less (by ~15%) in the presence of all pesticides, except mapanyperim. A lower effect (~5%) was found during the fermentative process with *L. plantarum*. Chemical treatments against fungi, such as mildew and *Botrytis* not only affect yeast but also lactic acid bacteria in wine, and delay MLF (Cabras *et al.*, 1994).

Vidal *et al.* (2001) examined the inhibitory effect of two commonly used pesticides, copper and dichlofluanid, on several strains of *O. oeni* and on MLF in simulated wine. Sensitivity to these pesticides varied and was enhanced by the presence of ethanol. Inhibition was due to a decrease in cell number and not to a decrease in malolactic activity. Quinoxyfen,

belonging to the family of quinolines, showed no effect on the MLF using *L. plantarum* (Cabras *et al.*, 2000). Ruediger *et al.* (2005) studied the effect of fungicides and insecticides on *O. oeni*. Chlorpyrifos and dicofol significantly reduced the activity of the lactic bacteria. Dicofol had a major inhibitory effect, while chlorpyrifos and fenarimol had a minor effect.

B. Effect of malolactic fermentation on pesticide residues

The influence of six fungicides (azoxystrobin, cyprodinil, fludioxonil, mepanipyrim, pyrimethanil, and tetraconazole) on two lactic bacteria (*L. oenos* and *L. plantarum*) was studied (Cabras *et al.*, 1999). During MLF, no degradative effect on pesticide levels was determined.

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