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Fenhexamid Adsorption Behavior on Soil Amended with Wine Lees

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The adsorption of fenhexamid (FEN) [*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide] on vineyard soil amended with wine lees (WL) produced by vinery was studied. The adsorption extent depends on WL fraction. The addition of the centrifuged solid lees (SWL) increases the FEN adsorption on soil. Most likely, the organic insoluble fraction formed mainly by dead fermentation yeasts is responsible for the observed increase. The adsorption measured on some deactivated yeasts of wine fermentation shows that *Saccharomyces cerevisiae* are the most active in FEN retention. On the other hand, the soil amendment with whole WL decreases considerably the fungicide adsorption. This opposite effect may be the result of FEN hydrophobic bonds with the dissolved organic matter of lees that keeps fungicide in solution. This hypothesis is substantiated by the increased FEN solubility in the supernatant of centrifuged wine lees (LWL). The results of soil column mobility confirm that the elution with LWL increases the mobility of FEN in soil.

KEYWORDS: Fenhexamid; adsorption; yeasts; DOM; wine lees; mobility; solubility

INTRODUCTION

Biosorption can be used for the removal of pollutants from environment. It can be defined as the passive uptake of xenobiotics by dead or inactive biological materials or by materials derived from biological sources (*1*). A variety of biomaterials are known to bind these pollutants, including agricultural wastes like wine lees. Wine lees are the residue formed, after fermentation, at the bottom of recipients containing wine during storage or after authorized treatments, as stated by the EEC regulation No. 337/79. Although the composition of lees is variable, they are composed mainly by yeasts, and, to a minor extent, by tartaric acid and inorganic matter (*2*).

The effects of winery wastewater soil amendment on plants and soil properties were studied. Lees treatment improves the nutritional status of soil (*3*) and does not exhibit toxicity risk for crops and the environment (*4*). Moreover, the lees ability in removing undesirable compounds from wine has been widely reported. Decreased amounts of volatile phenols were found in wines containing yeast lees as compared to the same wines aged without lees (*5*). De Melo Abreu et al. found that, during the vinification process, part of famoxadone fungicide residues is removed with the lees at the end of fermentation (*6*). Analogously, Navarro et al. reported that during the racking step six pesticides are adsorbed onto lees to an extent dependent on the given pesticide (*7*).

On the other hand, the effectiveness of wine lees in adsorbing soil organic contaminants has received much less attention (*8*). The addition of wine waste to vineyard soil affects the adsorption and leaching of the same fungicides, depending on their hydrophobic character (*9*). The wine industry is one of the most important industrial activities in Sardinia (Italy). Therefore, the addition of the wine lees to soil could be a suitable way for recycling organic matter and nutritive elements in the soil-crop system. FEN (**Figure 1**) is a hydroxyanilide fungicide widely used in grapevine crops exhibiting an excellent activity against *Botrytis cinerea* (*10*). In a study of ours carried out on the alcoholic fermentation of *Saccharomyces cerevisiae* in the presence of FEN, we observed a slight decrease of fungicide concentration in the fermentation medium (*11*). Missing FEN was recovered unchanged from yeasts. This suggested that missing fungicide was not degraded during the fermentation process, but rather adsorbed by yeasts. Two constituents of *S. cerevisiae* cell wall, chitin and glucan, tested as potential adsorbents, exhibited affinity for FEN (*11*).

This work was aimed at studying the influence of the amendment of vineyard soil with wine lees on FEN adsorption. To evaluate the role played by different yeasts, the study was extended to three deactivated wine yeasts (*Kloeckera apiculata*, *Metschnikowia pulcherrima*, *S. cerevisiae*) and to lees of laboratory fermentation carried out with a mixture of these yeasts.

MATERIALS AND METHODS

Materials. Fenhexamid (*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide), molecular weight 302.19, was supplied

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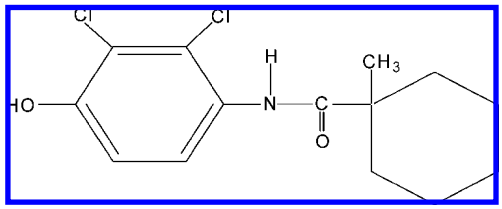

Figure 1. FEN chemical structure.

by Bayer, Milan, Italy. Its purity (99.2%) was checked by high performance liquid chromatography (HPLC).

All the solvents were of HPLC grade (Carlo Erba Reagenti, Milan, Italy) and were used without further purification.

Soil. A vineyard sandy loam soil from Sardinia, Italy, was examined. The sample was air-dried and sieved to <2 mm. The particle size distribution was measured by the Purdue University Soil Testing Laboratory using the pipet method (*12*). The organic carbon content was determined according to the modified Walkley−Black (*13*) method. The cation exchange capacity (CEC) was determined according to the procedure of Hendershot and Duquette (*14*). Soil pH was determined on slurries with a soil/water ratio of 1:1. Soil physicochemical properties were as follows: pH 7.3, 1.0% organic matter (OM), 11.0 cmol kg^{-1} CEC, 8.0% clay, 20.0% silt, and 72.0% sand.

Yeast. The strains *K. apiculat*a No 3197, *M. pulcherrima* No 606 and *S. cerevisiae* No 1090 were obtained from the collection of the Dipartimento di Scienze Ambientali Agrarie e Biotecnologie Agroalimentari, University of Sassari, Sassari, Italy. Precultures were prepared in broth containing 2% of glucose, 0.5% of yeast extract and 1% of peptone in a thermostatically controlled chamber at 25 °C for 48 h. Cells were washed twice and suspended in 0.15 M NaCl.

Laboratory Fermentation. Fermentation medium was made up containing 7 g L^{-1} of yeast nitrogen base (YNB) and 180 g L^{-1} of glucose at pH 3.6. The broth was sterilized by filtration through membrane filters (0.2-μm pore size) and then inoculated with a mixture of *K. apiculata*, *M. pulcherrima* and *S. cerevisiae* (50, 30, 20%, respectively). The amounts of inoculum were such as to ensure 1×10^6 cells mL^{-1} in the fermentation medium. The flask was put into a thermostatically controlled chamber at 25 °C for 12 days. At the end of fermentation, the suspension was centrifuged at 19000*g* for 20 min. The solid residue was inactivated by heating at 80 °C for 24 h in an oven.

Wine Lees. Two wine lees were used, namely, the residue of laboratory fermentation (SLL), obtained as described above, and the wine lees (WL) supplied by Sella e Mosca winery, Alghero, Italy. The wine lees from winery were centrifuged at 19000*g* for 20 min. The solid residue (SWL, 49.1% OM) was added to soil as an amending and the supernatant (LWL, 4.7% OM) was used in FEN solubility and mobility tests (see below). WL suspension from winery contained 31.6 mg solid mL^{-1}.

Solubility Test. Different amounts (40, 80 and 100 mg L^{-1}) of FEN exceeding its maximum solubility in water (20 mg L^{-1} at 20 °C) were added to 10 mL of LWL. The suspensions were equilibrated on a horizontal shaker at 20 °C for 24 h, then filtered to separate the fungicide excess. The amount of dissolved FEN was measured by HPLC.

Adsorption on Soil. Adsorption trials were carried out using the batch equilibration technique at 25 ± 2 °C. Lees were added to vineyard soil both as WL and as SWL. In general, 0.63 mL of WL or 20 mg of SWL was added to triplicate samples of 1 g of soil in polyallomer centrifuge tubes. After 24 h the samples were spiked with 2 mL of fungicide aqueous solution. FEN concentrations ranged from 8.3 to 33.0 μM. The tubes were shaken (end over end) for 15 h. After equilibration, the suspension was centrifuged at 19000*g* for 20 min and the supernatant was pipetted off and analyzed immediately. Adsorbed FEN was calculated from the difference between the initial and final concentrations of fungicide in solution.

A control adsorption experiment was prepared consisting of 1 g of natural soil, without any amendment, equilibrated with fungicide solution. The amount adsorbed was quantified with the same procedure described for amended soils.

Mobility on Soil Column. Experiments on soil columns were performed at room temperature. Glass columns of 20 cm × 1 cm i.d. were packed with 10 g of soil, and soil was saturated with 2 mL of LWL. A solution of 10 mg of FEN in 10 mL of acetone was added to 1 g of soil. Acetone was evaporated under vacuum, and the fortified soil was layered to column top. Then, the column soil was eluted with 8 pore volumes (about 32 mL) of LWL to reach a constant flow rate (4 mL h^{-1}). Column leachates (2 mL portions, 30 min) were collected. The FEN amount leached was tested by HPLC. A control adsorption experiment was performed as described above, but the column was saturated and eluted with deionized water.

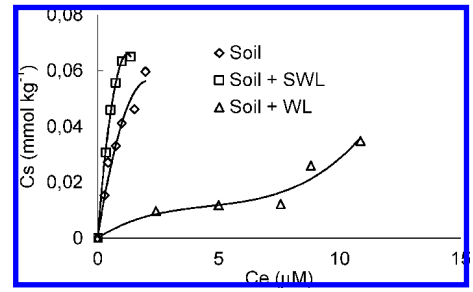

Figure 2. FEN adsorption isotherms on natural soil, on soil amended with SWL and soil amended with WL.

Adsorption on Wine Lees. Duplicate samples of 25 mg both of SWL from winery and of SLL were equilibrated in polyallomer centrifuge tubes with 2.5 mL of fungicide aqueous solution at 25 ± 2 °C. FEN concentrations ranged from 8.3 to 33.0 μM. The tubes were shaken (end over end) for 15 h. The amount adsorbed by different sorbents was quantified with the same procedure described for amended soil.

Adsorption on Deactivated Yeasts. Duplicate samples of 25 mg of deactivated yeasts were equilibrated in polyallomer centrifuge tubes with 2.5 mL of fungicide aqueous (8.3−33.0 μM) solutions at 25 ± 2 °C. The adsorption trials were carried out with the same procedure described for amended soil and wine lees.

HPLC Analysis. FEN concentration was determined by HPLC. A Waters 510 pump equipped with a 150 × 4.6 mm i.d. Spherisorb ODS (5 μm) analytical column, a multiwavelength Waters 2487 programmable detector operating at 230 nm and a Waters Breeze chromatography workstation were used. Acetonitrile plus water (50 + 50 by volume), previously brought to pH 2.7 with phosphoric acid, at a flux rate of 0.7 mL min^{-1} was the eluant. The retention time for FEN, under the chromatographic conditions described, was 11.3 min. The quantitative determination of FEN was based on external standards. Calculations were based on the average peak areas of the external standards. The detection limit for FEN was 0.01 mg L^{-1}, measured as the concentration of herbicide needed to obtain a detector response approximately twice the background signal.

Data Analysis. Adsorption data were fit to the logarithmic form of the Freundlich equation

$$\log C_s = \log K_f + 1/n \log C_e$$

where C_s (in mmol kg^{-1}) is the amount of fungicide adsorbed, C_e (in μM) is the equilibrium concentration in solution, and log K_f and $1/n$ are empirical constants representing the intercept and the slope of the isotherm, respectively. Fitting was performed by the least-squares regression analysis. The conformity of the sorption data to a linear isotherm was assumed when the correlation coefficient r was ≥0.97.

RESULTS AND DISCUSSION

Adsorption on Soil, SWL and Soil Amended with SWL. FEN adsorption was studied on three different systems: vineyard soil, SWL, and soil amended with SWL. The relative adsorption isotherms are shown in **Figures 2** and **3**. The calculated constants K_f and $1/n$ and the correlation coefficients (r) for the linear fit of the Freundlich equation are given in **Table 1**.

The adsorption isotherm of fungicide on vineyard soil shows a slope ($1/n$) less than 1, resembling the L-type curve described by Giles et al. (**Figure 2** (*15*)). This shape suggests a relatively

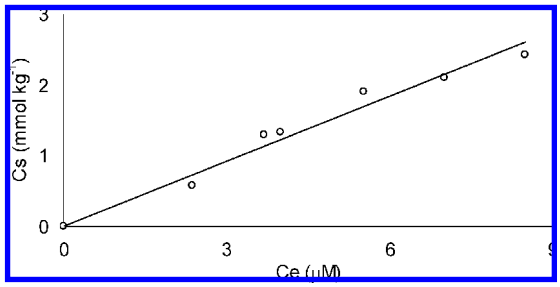

Figure 3. FEN adsorption isotherm on SWL.

Table 1. Freundlich Parameters for FEN Adsorption on Different Adsorbents[a]

adsorbent	$K_f(10^3 \mu mol^{(1-1/n)}L^{1/n}kg^{-1})$	1/*n*	*r*
natural soil	0.038 (±0.006)	0.63 (±0.03)	0.972
SWL	0.252 (±0.021)	1.11 (±0.02)	0.970
soil + SWL	0.055 (±0.011)	0.51 (±0.07)	0.972
soil + WL	0.005 (±0.002)	0.82 (±0.05)	0.996

[a] SD in parentheses.

high affinity of the herbicide for the adsorbing sites. FEN is a scarcely polar pesticide (log Kow = 3.51 (*16*)), therefore it is expected to exhibit a good affinity for nonpolar surfaces. Most likely, the interaction between FEN and organic matter of sandy loam soil is responsible for the adsorption observed.

SWL exhibits a remarkable affinity for fungicide. The adsorption isotherm of FEN on SWL, **Figure 3**, is a C-type curve (*15*) indicating a constant partition of solute between solution and adsorbent surface. This result agrees with the ability of yeast in retaining FEN already observed in a previous study (*11*).

When natural soil is amended with SWL, a light increase of FEN adsorption is observed and the isotherm is again of the L type. It is difficult to state if the increase is due to a previous adsorption of SWL organic components on the soil surfaces affording fresh hydrophobic surfaces available for fungicide adsorption. Alternatively, the greater organic matter content in the soil amended with SWL could be responsible for a higher adsorption of hydrophobic FEN. However, the vineyard sandy soil used is very poor in fractions suitable for adsorption, particularly clays. Moreover, the isotherm shape similarity for FEN adsorption on amended and nonamended soil suggests that analogous mechanisms take place. Therefore, we believe the second hypothesis more probable.

The biomass of lees consists mainly of yeasts that proliferate during the fermentation and die when nutrients are depleted. Industrial waste fermentation biomasses are excellent metal sorbents, and generally, the yeast adsorption ability is ascribed to their cell walls (*17*). On the other hand, in the literature, only little information is found concerning the adsorptive properties of wine fermentation yeasts toward the organic molecules (*18*−*20*) and in particular toward pesticide (*9*). In addition to *S. cerevisiae*, "the wine yeast", other non-*Saccharomyces* yeasts predominate in the first stages of wine fermentation. Among non-*Saccharomyces* yeasts, *K. apiculata*, and *M. pulcherrima* are the most frequent yeasts in fresh must (*21*). Therefore, we believed it to be of interest to evaluate the contribution to FEN adsorption on lees from each of these three yeasts.

Adsorption on Yeasts and on Solid Lees from Laboratory Fermentation. *K. apiculata*, *M. pulcherrima* and *S. cerevisiae* were inactivated before the adsorption test. The adsorption isotherms of FEN on inactivated yeasts cells are shown in **Figure 4**. On *S. cerevisiae* strain, the adsorption isotherm is

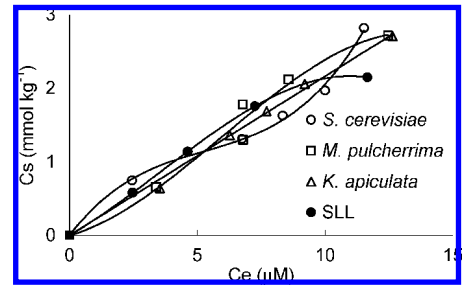

Figure 4. FEN adsorption isotherms on yeasts and on SLL.

Table 2. Freundlich Parameters for FEN Adsorption on Yeasts and SLL[a]

yeast	$K_f(10^3 \mu mol^{(1-1/n)}L^{1/n}kg^{-1})$	1/*n*	*r*
S. cerevisiae	0.360 (±0.048)	0.77 (±0.06)	0.970
K. apiculata	0.157 (±0.023)	1.14 (±0.05)	0.996
M. pulcherrima	0.176 (±0.025)	1.28 (±0.05)	0.983
SLL	0.287 (±0.019)	0.86 (±0.07)	0.985

[a] SD in parentheses.

convex, **Figure 4**, resembling the L3-type curve described by Giles et al. (*15*). The L shape suggests a relatively high affinity of the solute for the adsorbing sites. The subgroup 3 refers to the occurrence of a second rise suggesting the development of a fresh surface on which further adsorption can occur. Generally, this surface consists of the exposed parts of a solute layer already adsorbed. The adsorption isotherm of FEN on devitalized *K. apiculata* strain, **Figure 4**, is a C-type curve (*15*) or partition isotherm.

Finally, the adsorption isotherm on inactivated *M. pulcherrima* strain is of the S3-type (**Figure 4**). The S shape is indicative of an adsorption increasingly favored as the concentration of solute increases. Also in this case, the 3 subgroup is explained by the development of a new hydrophobic adsorbent surface consisting of adsorbed FEN molecules which enhance the adsorption of fungicide molecules through hydrophobic bonding. It is worth noting that the isotherms on inactivated *S. cerevisiae* and *M. pulcherrima* yeasts show, at the highest C_e values, comparable C_s values (**Figure 4**). This means that, for both yeasts, surfaces with similar adsorptive capacity are involved in the second adsorption rise. Indeed, after the fungicide has filled yeast adsorption sites, further adsorption takes place on the new surface formed only by adsorbed FEN molecules. The calculated constants K_f and 1/*n*, and the correlation coefficients (*r*) for the linear fit of the Freundlich equation are reported in **Table 2**. K_f values indicate that *S. cerevisiae* exhibits the highest adsorption, followed by *M. pulcherrima* and *K. apiculata*.

The yeast adsorption ability is ascribed to cell wall components (*22*). Among them, β-D-glucan plays a major role in biosorption, whereas chitin is not very effective (*11, 23, 24*). Most likely, the different behavior may be ascribed to the different ratio between cell wall constituents. In fact, the adsorption activity is notably different from yeast to yeast, according to structural characteristics and chemical composition of the outermost layer of cell wall (*25*).

To state if FEN adsorption on SWL is mainly the result of a fungicide interaction with dead yeast present on winery lees, an adsorption test on SLL coming from laboratory fermentation was carried out.

The laboratory fermentation was inoculated with a mixture of *K. apiculata*, *M. pulcherrima* and *S. cerevisiae* (50, 30, 20%, respectively). This distribution corresponds approximately to the status at the beginning of natural fermentation. At the end

Table 3. FEN Solubility in Water and in LWL[a]

FEN (mg mL^{-1})	water (mg mL^{-1})	LWL (mg mL^{-1})
0.02	0.016 (±0.006)	0.021 (±0.002)
0.04	0.020 (±0.002)	0.033 (±0.012)
0.08	0.017 (±0.004)	0.048 (±0.011)
0.10	0.023 (±0.003)	0.060 (±0.008)

[a] SD in parentheses.

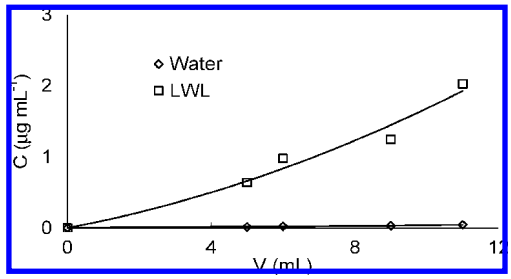

Figure 5. FEN leaching in soil column eluted with LWL or water.

of fermentation, the suspension was centrifuged and the solid residue was inactivated. The respective Freundlich parameters are reported in **Table 2**. The adsorption observed on SLL is not much different than that on SWL, which indicates that yeasts are the main sorbents in lees.

Adsorption on Soil Amended with WL. The addition of the whole winery suspension lees to soil lowers 8-fold the adsorption of fungicide (**Table 1** and **Figure 2**). K_f decreases from 0.04 for the natural soil to 0.005 for the soil handled with WL. The incorporation of organic amendments introduces dissolved organic matter (DOM) into soil. The addition of DOM to soil can increase or decrease the pesticide adsorption depending on the chemical-physical properties of the xenobiotic (*9, 26*−*28*). Generally, with hydrophobic molecules like FEN, DOM reduces the adsorption and/or increases the desorption due to stable interactions with pesticide in solution, or competing with the pesticide molecules for adsorption sites on the soil surfaces. To understand why DOM decreases FEN adsorption on soil amended with WL, a solubility test was carried out. FEN solubility in water and in LWL is reported in **Table 3**. The doubled or tripled fungicide solubility in LWL indicates that interaction occurs between DOM and fungicide in solution. This confirms greater FEN affinity to DOM in LWL than to soil surface. A potential effect of such interaction is the enhancement of FEN transport by DOM through soil profile.

Mobility Experiments. Mobility experiments were performed on soil columns using LWL as an eluant and deionized water as a control (**Figure 5**). FEN is fast leached in soil column eluted with LWL: the fungicide is found in the leachate collected after 1.25 h (5 mL); on the other hand, the water elution shifts the detection of FEN to 6 h (24 mL). These results clearly emphasize the role of DOM in promoting leaching of hydrophobic compounds.

The results of this work show that SWL increase FEN adsorption on soil due to ability of insoluble organic matter, mainly yeast wall cells, in retaining the fungicide. Nevertheless, DOM present in WL overcomes the role of the insoluble constituents in enhancing fungicide adsorption and, consequently, increases the risk of groundwater contamination.

ABBREVIATIONS USED

FEN, fenhexamid; WL, wine lees; SWL, centrifuged solid lees; LWL, supernatant of centrifuged wine lees; SLL, solid residue of laboratory fermentation; DOM, dissolved organic matter.

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