Effects of Diffuse Colonization of Grape Berries by *Uncinula necator* on Bunch Rots, Berry Microflora, and Juice and Wine Quality

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ABSTRACT

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Production of grape (principally cultivars of *Vitis vinifera*) for high-quality wines requires a high level of suppression of powdery mildew (*Uncinula necator* syn. *Erysiphe necator*). Severe infection of either fruit or foliage has well-documented and deleterious effects upon crop and wine quality. We found that berries nearly immune to infection by *U. necator* due to the development of ontogenic resistance may still support diffuse and inconspicuous mildew colonies when inoculated ≈ 3 weeks post-bloom. Fruit with diffuse mildew colonies appear to be

healthy and free of powdery mildew in late-season vineyard assessments with the naked eye. Nonetheless, presence of these colonies on berries was associated with (i) elevated populations of spoilage microorganisms; (ii) increased evolution of volatile ethyl acetate, acetic acid, and ethanol; (iii) increased infestation by insects known to be attracted to the aforementioned volatiles; (iv) increased rotting by *Botrytis cinerea*; and (v) increased frequency of perceived defects in wines prepared from fruit supporting diffuse powdery mildew colonies. Prevention of diffuse infection requires extending fungicidal protection until fruit are fully resistant to infection. Despite a perceived lack of improvement in disease control due to the insidious nature of diffuse powdery mildew, potential deleterious effects upon crop and wine quality thereby would be avoided.

Production of wine grape, principally cultivars of the European species *Vitis vinifera* L., requires a high level of suppression of powdery mildew (*Uncinula necator*, syn. *Erysiphe necator*). Degradation of crop and wine quality by high levels of powdery mildew on either foliage or fruit has been documented in previous studies (1,5,7,17,29,31,33). Severe foliar infection can debilitate vines, reduce net photosynthesis, and retard ripening and sugar accumulation (5,17,31), all of which can decrease the quality of the affected crop.

Most but not all studies have indicated that very low levels of powdery mildew on fruit can degrade the quality of wine. Inclusion of as little as 3% (wt/wt) of severely infected berries yielded perceived defects in red wines prepared from the *Vitis* interspecific hybrid cv. Rosette (31). Ough and Berg (29) prepared red and white varietal wines from *V. vinifera* 'Thompson Seedless', 'Carignane', or 'Ribier' grape berries with or without "visual mildew infections" and wines prepared from grape berries without visual mildew were ranked as preferable to those prepared from berries bearing visual mildew in 75 to 88% of samplings by a panel of tasters. Stummer et al. (33) reported compositional and sensory degradation of juice and white wines prepared from *V. vinifera* 'Chardonnay' fruit when >1 to 5% of the bunches were affected by powdery mildew. However, Calonnec et al. (5) reported that powdery mildew had no significant impact on perceived quality of red wines prepared from *V. vinifera* 'Cabernet

Sauvignon' until the weight of mildewed fruit exceeded 25% of the total harvested. An even higher threshold (i.e., $>50\%$ mildewed fruit, by weight) was reported in this study (5) as the minimum level producing a detectable effect in sensory evaluations of white wines prepared from berries of *V. vinifera* 'Sauvignon Blanc'.

During the course of previous investigations of ontogenic resistance to powdery mildew in grape berries, we found that berries infected just prior to development of immunity supported diffuse, inconspicuous, nonsporulating colonies (12,13,15). These colonies were not noticeable in vineyard assessments of the disease, even when $\geq 30\%$ of the berries within a cluster were so affected. In subsequent experiments, we noted that fruit supporting diffuse powdery mildew often became severely rotted at harvest due to natural infections by *Botrytis cinerea*. Our present report is based upon the results of extensive vineyard and winery studies wherein we document the deleterious effects of diffuse powdery mildew on crop and wine quality, and elucidate the possible mechanisms by which this degradation occurs through the agency of *B. cinerea*, fruit-feeding insects, and the epiphytic microflora of diseased berries. Preliminary accounts of this work have been published (16,18,19).

MATERIALS AND METHODS

Impact of diffuse powdery mildew on bunch rot, insect infestation, evolution of fruit volatiles, and berry microflora. Studies were conducted during the 1999 and 2000 growing seasons in a previously described (15) mixed planting of *V. vinifera* cultivars at Geneva, NY and in a 10-ha commercial vineyard of *V. vinifera* 'Pinot Noir' at Lodi, NY, during the 1999 to 2002 growing seasons. Two treatments were established in all tests, and were replicated four times in three-vine plots that were located

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randomly within the vineyards. In the first treatment, designated “healthy,” powdery mildew and other fungal diseases were controlled rigorously by regular fungicide applications, beginning at 15 cm of shoot growth and continuing at 7- to 14-day intervals until veraison. In the second treatment, designated “diffuse powdery mildew” (DPM), 30 clusters per replicate were inoculated with a suspension containing *U. necator* at 10^5 conidia/ml, as described by Gadoury et al. (14), ≈ 3 weeks after the completion of bloom and midway between two fungicide applications. Our previous research (12,13,15) had shown that berries of multiple *V. vinifera* cultivars become highly resistant, but not immune, to infection and subsequent pathogen development at this stage of crop development.

Bunch rot severity as well as apparent (easily visible) incidence and severity of powdery mildew were assessed at harvest in each year of the study. In addition, the percentage of clusters infested with ants (Formicidae: *Leptothorax* spp.), picnic beetles (*Glischrochilus quadrisignatus*), and yellowjackets (*Vespula* spp.) also was recorded on Pinot Noir clusters at Lodi in 2001 and 2002. Ten clusters per replicate then were picked, brought to the laboratory, and examined at $\times 25$ magnification for evidence of diffuse powdery mildew colonies. Nonsporulating diffuse colonization of grape berries by *U. necator* is rarely visible to the naked eye (15), but can be resolved with a fiber-optic illuminator directed tangentially to the berry surface. Ten berries were picked from each of the sampled clusters and examined as above. The percentage of the berry surface supporting diffuse powdery mildew colonies was estimated visually and recorded. Feeding behavior of the above insects was observed on several occasions at the Lodi site, and upon selected clusters returned to the laboratory in 2001 and 2002.

Among the Pinot Noir clusters harvested at the Geneva and Lodi site in 2000, and at the Lodi site in 2001 and 2002, 10 berries from each of 10 clusters per treatment replication were shaken for 30 min in a 1-liter Erlenmeyer flask containing 100 ml of an extraction buffer (9 g of NaCl, 1 g of peptone, and 100 μ l of sodium dodecyl sulfate per liter). Four semiselective agar media in 9-cm petri plates were employed to quantify the berry microflora: (i) malolactic broth agar plus cyclohexamide at 300 μ g/ml (MLBC), for growth of lactic acid bacteria (6); (ii) yeast medium agar (YMA), for growth of yeast genera commonly found on grape berries and in wine (2); (iii) yeast medium agar plus cyclohexamide at 300 μ g/ml (YMC), for growth of cyclohexamide-resistant yeasts, primarily *Brettanomyces* (3); and (iv) lysine agar (LYS) for growth of selected non-*Saccharomyces* yeasts, such as *Hanseniaspora*, *Aureobasidium*, *Metschnikowia*, and *Candida* spp. (34). A 100- μ l aliquot of the crude extract and 100- μ l aliquots of 10^{-1} , 10^{-2} , and 10^{-3} dilutions of the crude extract were dispensed into petri plates of each medium and spread on the surface using a glass rod. The plates were incubated at 25°C for 6 days, and then the number of colonies was recorded for the dilution that produced at least 50 discrete colonies per plate.

For fruit collected from both sites in 2000, 30 colonies were selected at random from each of three replicate plates of each medium, subcultured, and identified, insofar as possible, based upon their morphology at $\times 400$ to 800. In general, this allowed only the tentative identification of yeast genera (*Saccharomyces*, *Candida*, *Metschnikowia*, *Brettanomyces*, *Kloeckera*, *Aureobasidium*, and *Hanseniaspora*) and the bacterial genera *Acetobacter* and *Lactobacillus*. Additional and more definitive polymerase chain reaction (PCR) and sequencing methods were used to identify yeast genera that were isolated on YMA and LYS from fruit at Lodi in 2001 and 2002. Twenty colonies selected at random from each replicate plate per medium were subcultured and identified. Cultures grown from single cells were suspended in 10 mM Tris-HCl buffer (pH 8), and lysate was obtained by boiling the cells in microfuge tubes for 10 min. Cell debris then was removed by centrifugation at $14,000 \times g$ and the supernatant

removed to a clean microfuge tube for storage at 4°C. Primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GTCCGTGTTTCAAGACGG-3'), specific for a portion of the large subunit (LSU) of the 26S rDNA gene of ascomycetous yeasts, were synthesized based on sequences from a previously published method (25,27), and were used in 50- μ l Hot Start Taq MasterMix (Qiagen, Chatsworth, CA) with reagents at the kit-recommended concentrations. The reaction was performed in a Stratagene Gradient-Plus thermal cycler. PCR reactions were carried out after one cycle of incubation for 15 min at 95°C. Amplification of the 26S rDNA fragment was accomplished in 40 cycles of denaturation at 95°C for 50 s, annealing at 52°C for 50 s, and elongation at 72°C for 2 min. A final step of 72°C for 10 min was part of the programs. Amplification products were analyzed via electrophoresis at 100 V for 60 min in a 1% agarose gel stained with ethidium bromide following standard protocols (32). PCR products were then purified and concentrated for sequencing by using the QiaQuick PCR purification kit (Qiagen). Elution from the spin column with 30 to 50 μ l of water concentrated the amplification product to the appropriate level required for sequencing following the standard methods used by the Cornell University BioResource Center Sequencing Facility. Purified and concentrated PCR product was quantified and mixed with the same primers used for PCR. Approximately 1 μ g of amplicon (10 μ l) was mixed with 8 pmol of either forward or reverse primer. Sequencing reactions were carried out at the BioResource Center for Advanced Technology at Cornell University and separated on an Applied Biosystems Automated 3730 DNA Analyzer (ABI, Foster City, CA). Results from both strand sequences were assembled and the resultant sequence analyzed with the DNASTar analysis software package (Lasergene, Madison, WI). DNA consensus sequences of the portion of the LSU of the 26S rDNA gene were submitted to a BLAST search on the National Center for Biotechnology Information website.

Volatiles evolved by berries with and without diffuse powdery mildew. Three replicate samples of five Pinot Noir clusters each were collected from the healthy and DPM treatments at the Lodi site on the commercial harvest date in 2001, 2002, and 2003 and were sealed immediately in 6-liter polyvinylchloride boxes. Volatile ethyl acetate, acetic acid, and ethanol evolved by the clusters were assayed by solid-phase microextraction (SPME) as described by Pawliszyn (30), and by gas chromatography/mass spectroscopy (GCMS). A 2-cm-long three-phase extraction polymer SPME fiber with affinity for low molecular weight volatiles was exposed for 30 min to extract volatiles from the headspace above the respective grape clusters. The SPME fiber was thermally desorbed at 250°C in a splitless injection into an Agilent 6890 gas chromatograph equipped with an Agilent 5973 Mass Selective Detector (Agilent Technologies, Wilmington, DE) and HP-5MS capillary chromatography column (Agilent Technologies). Ion peak areas for the three target analytes were integrated and recorded. Grape clusters were assayed in this manner at 24-h intervals up to 72 h after harvest.

Impact of fruit-feeding insects on severity of bunch rot on fruit clusters with or without diffuse powdery mildew. Wounds created by fruit-feeding insects are potential infection courts for microorganisms that cause bunch rots. At the Lodi site in 2001 and 2002, a subset of 30 clusters from each replicate of the healthy and DPM treatment was sprayed with the insecticide fenpropathrin (Danitol 2.4 EC; Valent Biosciences Corp., Walnut Creek, CA) at 0.5 g/liter a.i. at veraison, and twice more at 10-day intervals in order to deter infestation of the clusters by fruit-feeding insects. No other fruit were treated with an insecticide. Incidence of insect infestation and the severity of bunch rot were recorded at harvest in all treatments.

Preparation and evaluation of wines from berries with and without diffuse powdery mildew. Plots consisting of five rows with 100 vines each were replicated three times for both the

Fig. 1. **A**, Mildew-free Chardonnay fruit cluster (left) and fruit cluster in which 24% of the berries support one or more diffuse powdery mildew colonies (right). Macroscopically, both clusters appear free of powdery mildew. **B**, Nonsporulating hyphae from diffuse powdery mildew colony. **C**, Cross section of Chardonnay berry epidermis showing necrosis of two epidermal cells (nec) beneath hyphae of diffuse powdery mildew colony. **D**, *Botrytis cinerea* emerging from lenticel (left) and calyx end (right) of Chardonnay berry bearing diffuse powdery mildew colonies.

healthy and DPM treatments at the Lodi site in 2000, 2001, and 2002. Vines in the healthy treatment were sprayed rigorously with fungicides per the grower's standard program to control powdery mildew and other fungal diseases. Within the DPM plots, all clusters were inoculated with a suspension of 10^5 conidia of *U. necator* per milliliter on a date midway between two fungicide applications, ≈ 3 weeks after bloom. Grape berries were harvested at ripeness in each year of the study. Approximately 110 kg of fruit were harvested from each plot, cooled to 4°C, and destemmed, and the sugar content measured with a refractometer. Destemmed berries were transferred to 120-liter stainless steel open top drums. To the crushed grape berries, SO_2 at 30 mg/liter and diammonium hydrogen phosphate at 1 g/liter was added. A 50-ml juice sample was collected and frozen for later analysis. Grape must was warmed to 25 to 30°C, then inoculated with a rehydrated yeast starter culture and fermented at 20°C on the skins to dryness (residual sugar at <2 g/liter). The wine was pressed and filled into 20-liter glass carboys fitted with fermentation locks. At the end of alcoholic fermentation, the wine was inoculated with a malolactic starter culture (Lallemand OSU-1) and allowed to complete malolactic fermentation at 20°C. It then was racked and sulfited (20 $\mu\text{g/ml}$), racked again if necessary, with free SO_2 adjusted to 20 to 30 mg/liter before cold stabilization at -2°C for 2 to 4 weeks. After cold stabilization, the wine was racked again (with free SO_2 adjusted to 30 mg/liter), bottled into 750-ml bottles with cork closures, and stored at 16°C for a minimum of 18 months before sensory evaluation. Juice samples were analyzed for pH, titratable acidity (TA), and total soluble solids ($^\circ\text{Brix}$). Malic, lactic, tartaric, and acetic acid were measured by high-performance liquid chromatography, and color was measured by absorbance at 280, 420, and 520 nm. Wine samples were analyzed for pH, TA, free/total SO_2 , malic, lactic, tartaric, and acetic acid, residual sugar (glucose + fructose), and color.

Wines were evaluated by a panel of 8 to 11 individuals in a blind test where samples were assigned three-digit codes (26). The wines were served in ISO standard wineglasses at room temperature (22 to 25°C) in random order, and each individual rated each replicate twice. Individuals ranked each replicate for intensity of the following standard organoleptic factors on a nonstructured 10-cm line scale (26) denoting intensity of (i) cherry or berry flavor, (ii) pepper or spice, (iii) green or vegetative taste, (iv) mouth feel, and (v) bitterness. In cases where an individual perceived a negative attribute in the taste or aroma of a replicate, this attribute also was described and recorded in tasting notes. To standardize rankings based upon the sensitivity of individuals in scoring the above factors, all rankings for a given factor determined by an individual were scaled to the maximum value applied to that factor by the same individual. For example, if an individual assigned a maximum value of 8.5 across all wines for the factor "bitterness," all other values recorded by that individual for bitterness were standardized as a fraction of 8.5, and these resultant values were used to compute treatment means and standard errors. Negative attributes that were perceived by an individual in both the first and second sampling of a replicate were compiled and the percentage of individuals on a panel that applied one or more negative attributes to a replicate was recorded.

RESULTS

Diffuse colonization of grape berries was never visually evident in any vineyard assessment of disease 2 months after inoculation (Fig. 1A). However, examination of the berries at $\times 25$ magnification 3 weeks or more after inoculation revealed a diffuse and nonsporulating network of hyphae (Fig. 1B) that was accompanied by necrotic epidermal cells at the sites of appressorial attachment (Fig. 1C). From 24 to 58% of the berries in inoculated clusters

bore some diffuse powdery mildew colonies (Figs. 2 to 4), but the proportion of the berry surface bearing such colonies was relatively low, ranging from ≈ 1 to 10% (Figs. 2 to 4).

There was a clear association between the presence of diffuse powdery mildew and the severity of bunch rot (Fig. 1D) in all cultivars, locations, and years of the study (Figs. 2 to 4). Uninoculated fruit showed only trace levels of powdery mildew and were relatively unaffected by bunch rot (Fig. 2 to 4). In contrast, approximately one-fourth to one-half of the fruit inoculated with *U. necator* 3 weeks post bloom bore diffuse powdery mildew colonies, and bunch rot severity was manyfold greater ($P = 0.05$) on these clusters than on those that were not inoculated (Figs. 2 to 4).

Pinot Noir fruit with diffuse powdery mildew also were more heavily infested with ants (*Leptothorax* spp.), sap beetles (*Glischrochilus quadrisignatus*), and yellowjackets (*Vespula* spp.) (Fig. 5A to C) in both 2001 and 2002. Only trace percentages of diffuse powdery mildew occurred in the healthy treatment (Fig. 4; Table 1), and $<3\%$ of the clusters were infested by one or more of the above insects (Table 1). In comparison, from 27.3 to 34.8% of the berries from the DPM treatment bore diffuse mildew colonies in 2001 and 2002, respectively (Fig. 4), and the mean level of insect infestation ranged from 38.3 to 49.4% (Table 1) among clusters not treated with the insecticide fenpropathrin. Both the incidence of insect infestation and severity of bunch rot was significantly ($P = 0.05$) reduced when the insecticide fenpropathrin was applied to DPM clusters (Table 1). DPM clusters treated with fenpropathrin in 2001 were free of fruit-feeding insects when examined near harvest, and the incidence of bunch rot was reduced to 5.9%, compared with 15.9% in DPM clusters that were not treated with fenpropathrin (Fig. 4A; Table 1). Similar results were obtained in 2002 (Table 1).

Yellowjackets were observed while feeding upon DPM clusters in the vineyard (Fig. 5C and D), and upon clusters returned to the laboratory for microscopic observation (Fig. 5E and F). Feeding wounds usually were inflicted by yellowjackets within 30 s of being placed on intact fruit. The insect's mandibles produced slits ≈ 0.2 mm long in the berry epidermis, which then exuded sap from the internal tissue of the berry (Fig. 5F). Prolonged feeding (>5 min) resulted in multiple wounds of this type and eventual mining of a cavity extending into the internal tissues of the berry (Fig. 5A).

Fruit with DPM produced far more volatiles than healthy fruit when measured within 24 h after harvest (i.e., 13 to 259 times more volatile ethyl acetate, 4 to 18 times more volatile ethanol, and one to eight orders of magnitude more acetic acid) (Table 2).

Populations of epiphytic fungi and bacteria recorded on four different semiselective media were one to several orders of magnitude higher ($P = 0.05$) on berries with diffuse powdery mildew than on healthy fruit in all years of the study (Fig. 6A to D). The methods employed in 2000 (semiselective media with subsequent identification based on colony characteristics and microscopic examination) revealed no significant ($P = 0.05$) qualitative differences between DPM and healthy fruit in the distribution of the particular classes of epiphytic microbes (as reflected by their numbers on the specific media) as a percentage of total microflora at either Geneva or Lodi (Fig. 6A and B). However, more definitive PCR-based tests used in 2001 and 2002 to better identify the organisms recovered on YMA and LYS media indicated a significant ($P = 0.05$) change from a predominance of *Aureobasidium* spp. on healthy fruit (65.7 and 67.7% of total microflora in 2001 and 2002, respectively) to a predominance of *Hanseniaspora* spp. (68.8 and 80.8%, respectively). *Metschnikowia* sp. and *Candida stellata* also were detected on DPM berries in both years, but not on healthy fruit (Fig. 7). No significant ($P = 0.05$) differences were detected in either 2001 or 2002 in the comparative percentages of other species of *Candida*, *Sporidiobolus*, *Cryptococcus*, *Debaryomyces*, *Rhodotorula*, or *Acetobacter* isolated from healthy versus DPM fruit (data not shown). The pres-

Fig. 2. Effects of diffuse powdery mildew infection of grape berries upon the severity of Botrytis bunch rot at **A to D**, Geneva or **E**, Lodi, NY in 1999. Clusters at each location were subjected to the same fungicide spray program. “Healthy” clusters were relatively mildew free. “Diffuse infection” treatment was inoculated with a spore suspension of *Uncinula necator* midway between two fungicide sprays, just before berries became immune to powdery mildew infection (≈ 4 weeks post bloom). Severity of bunch rot was assessed 1 week preharvest. Numbers above each bar indicate the percentage of berries in clusters with any amount of diffuse powdery mildew, or (in parentheses) the percentage of the berry surface covered by diffuse powdery mildew. Bars indicate one standard error of the mean.

Fig. 3. Effects of diffuse powdery mildew infection of grape berries upon the severity of Botrytis bunch rot at **A to D**, Geneva or **E**, Lodi, NY in 2000. All clusters at each location were subjected to the same fungicide program. "Healthy" clusters were relatively mildew free. "Diffuse infection" treatment was inoculated with a spore suspension of *Uncinula necator* midway between two fungicide sprays, just before berries became immune to powdery mildew infection (≈ 4 weeks post bloom). Severity of bunch rot was assessed at 1 week preharvest. Numbers above each bar indicate the percentage of berries in clusters with any amount of diffuse powdery mildew, or (in parentheses) the percentage of the berry surface covered by diffuse powdery mildew. Bars indicate one standard error of the mean.

ence of DPM had no significant ($P = 0.05$) effect, following malolactic fermentation, on soluble solids levels of juice at harvest, or on pH, titratable acids, tartrate, malate, lactate, or acetate levels in wine made from these fruit, in any year of the study (Table 3). Neither were individuals able to detect significant differences ($P = 0.05$), following malolactic fermentation, between wines made from fruit with or without DPM with respect to the intensity of the organoleptic factors "cherry or berry flavor," "pepper or spice," "green or vegetative taste," "mouth feel," or "bitterness" during blind tastings. However, in all years of the study, individuals applied a significantly ($P = 0.05$) higher number of negative descriptors to wines prepared from fruit with DPM than to wines prepared from fruit essentially free of such infections (Fig. 8).

DISCUSSION

In previous work (12–15), we demonstrated that grape berries acquire substantial ontogenic resistance to *U. necator* ≈ 3 weeks after bloom. However, berries infected during the transition from moderate susceptibility to a high level of resistance develop the peculiar phase of the disease that we have called diffuse powdery mildew (15,18,19). This phase is typified by insidious mildew colonies that are not readily visible to the naked eye in vineyard assessments (Fig. 1A), and very likely would be missed in field surveys for powdery mildew, even by experienced observers. Diffuse powdery mildew represents a colony-level reaction by the dying pathogen to the gradual development of ontogenic resistance. It involves all of the processes noted in our earlier studies of ontogenic resistance (12–15), including reduced rates of hyphal growth, increased intensity of appressorium production, death of individual hyphal segments, and the death of individual epidermal cells underlying appressoria. The end result is a wounded but macroscopically unblemished epidermis. Even under a magnification of $\times 30$, the close appression of hyphae to the highly reflective

Fig. 4. Effects of diffuse powdery mildew infection of grape berries upon the severity of Botrytis bunch rot in a vineyard of *Vitis vinifera* 'Pinot Noir' at Lodi, NY in **A**, 2001 and **B**, 2002. All vines were subjected to the same fungicide spray program. "Healthy" clusters were relatively mildew free. "Diffuse infection" treatment was inoculated with a spore suspension of *Uncinula necator* midway between two fungicide sprays, just before berries became immune to powdery mildew infection (≈ 4 weeks post bloom). Severity of bunch rot was assessed 1 week preharvest. Numbers above each bar indicate the percentage of berries in clusters with any amount of diffuse powdery mildew, or (in parentheses) the percentage of the berry surface covered by diffuse powdery mildew. Bars indicate one standard error of the mean.

cuticle of the berry, the lack of sporulation, and the dehydrated condition of the dead hyphal segments that constitute much of a diffuse mildew colony make it difficult to view. However, hyphae were viewed under a dissecting microscope using oblique illumination, directing light across the mildew colony at a very shallow angle to reveal shadow details and avoid glare off the cuticular waxes (Fig. 1B).

Although the development of patches of necrotic epidermal cells (Fig. 1B and C) might provide a court for direct infection by *B. cinerea*, we lack conclusive evidence that this is the principal avenue by which diffuse powdery mildew increases the severity of Botrytis bunch rot. Sporophores of *B. cinerea* were observed emerging occasionally from patches of dead epidermal cells, but also from lenticels and the calyx and stem end of the fruit (Fig. 1D). Furthermore, the increased evolution of certain volatiles by fruit with diffuse infection and their associated infestation by fruit-feeding insects, accompanied by the decreased severity of bunch rot when such insects were largely excluded from fruit with

diffuse powdery mildew by applications of an insecticide, suggests that a more complex process may sometimes be involved. Fruit with diffuse powdery mildew emitted increased levels of ethanol, acetic acid, and ethyl acetate before there were visible signs of rot in the cluster, and all of these volatiles are used by a number of fruit-feeding insects as sensory cues to locate sources of food (23,24). At least one of the insects we found (*Vespula* sp.) could rapidly inflict wounds in the berry epidermis, which also might have provided an infection court for *B. cinerea*. Additionally, one or more of these insects may have physically dispersed conidia of *B. cinerea* to their feeding sites, as has been shown for larvae of the Lepidopterous insect, *Lobesia botrana* (11).

Fruit with diffuse powdery mildew has the potential to bring large numbers of deleterious bacteria and yeast into the grape must and, thereby, into wine fermentation. The common enological practice of adding SO₂ and a *Saccharomyces* yeast starter culture should largely suppress the further activity of these

Fig. 5. Insects differentially attracted to grape berries bearing diffuse powdery colonies. **A**, Ants (*Leptothorax* sp.) congregating around cavity excavated during feeding by yellowjackets (*Vesper* spp.). **B**, Sap beetle (*Glischrochilus quadrisignatus*) on Pinot Noir berry. **C** and **D**, Yellowjackets preparing to feed upon berries with unbroken skin; and **E**, damage to Pinot Noir berry inflicted by single yellowjacket during 30 s of feeding. Wound cavity (wc) is ≈ 1.0 mm in diameter and extends through the epidermal layer into the berry pulp. **F**, Exudation of sap from wound cavity in Pinot Noir berry after 30 s of feeding by a single yellowjacket.

numerous introduced organisms, but perhaps not before there was an impact on the sensory quality of the resulting wine. Our data indicate both a much larger overall load of non-*Saccharomyces* yeast and bacteria entering the winemaking process on fruit with DPM and a shift in the predominate genera from *Aureobasidium* on healthy fruit to *Hanseniospora*, *Candida*, and *Metschnikowia* on these diseased fruit. Although many of these yeast and bacteria die during the early stages of fermentation, certain genera predominating on fruit with diffuse powdery mildew (i.e., *Hanseniospora* and *Candida*) have a particularly high spoilage potential and can produce large amounts of acetic acid and acetate esters, as well as very potent and disagreeable flavors and odors that have been variously described as “fusel oil” or “cauliflower.” None of the genera identified on either healthy or DPM fruit are generally recognized as risks to human health, nor was our study a comprehensive survey of the microflora of healthy or diseased fruit. Further research will be required to determine the geographic scope of the relationships we noted between diffuse powdery mildew, bunch rot, fruit-feeding insects, and wine quality. However, the general effects we observed could be widespread.

Previous studies on relationships between powdery mildew and juice or wine quality generally have focused upon (i) reduced vine function that carried over into reduced fruit quality parameters, or (ii) a direct relationship between severity of fruit disease or *U. necator* biomass and wine quality (5,7,17,29,31,33). The levels of foliar powdery mildew in our study were nil; thus, the lack of any impact upon common fruit and juice quality parameters (Table 3) was not surprising. Our sensory evaluations of wines did not detect differences between wines with respect to those aspects of wine quality that primarily reflect optimal fruit maturity at harvest. Neither is the absolute quantity of *U. necator* biomass on fruit with diffuse powdery mildew comparable with that reported in studies dealing with visible and often severely diseased fruit (7,29,31,33). Our results do not confirm or refute these prior studies, which dealt with quantitatively and qualitatively different forms of grape powdery mildew, but rather address a form of symptom expression and disease effects that previously were unreported. We found no single consistent or characteristic flaw in wines prepared from fruit with diffuse powdery mildew; however, the aggregate number of defects was substantially higher among such wines than in those without diffuse infection (Fig. 8). It should be noted that inconsistent and even rare defects are still of great concern in the production and marketing of fine wines and that such defects might prove economically costly should they negatively impact consumer perception of wine brands.

The insidious nature of diffuse powdery mildew has important ramifications with respect to the practical management of the disease and the design of management programs. Specifically, disease management programs are developed primarily from trials in which visual ratings of fruit in the vineyard are the principal response by which efficacy of a program is judged. Such an approach may lead to the development of programs that effectively and reliably suppress *apparent* disease, but provide inconsistent and unreliable suppression of diffuse powdery mildew. Although the degree to which such a risk may be realized remains unknown, our present work demonstrates several negative consequences for producing fruit that appear to be free of powdery mildew, but which harbor diffuse colonies.

Another aspect of diffuse powdery mildew relates to its exacerbation of fruit rot by *B. cinerea*. Diffuse powdery mildew could represent a confounding factor in the performance of management programs for Botrytis bunch rot and a presently unconsidered variable in *Botrytis* forecast models. It is interesting that the highly refined petroleum oil product, JMS Stylet Oil (JMS Flower Farms, Inc., Vero Beach, FL), has been reported in some trials on cultivars highly susceptible to powdery mildew to reduce the severity of Botrytis bunch rot (8). However, efficacy trials conducted in New York indicate that although JMS Stylet Oil is highly effective against all phases of powdery mildew, it has shown no direct efficacy against Botrytis bunch rot (35–37). This contrast raises the possibility that the observed reduction of bunch rot by the oil in some trials may occur primarily through improved control of powdery mildew on fruit, in particular the diffuse colonization phase. Thus, the control of diffuse powdery mildew may be essential to preserving the integrity of fruit clusters during a period of high risk for bunch rot.

In our experiments, diffuse powdery mildew was initiated by purposefully inoculating fruit clusters midway between two fun-

TABLE 2. Relative increase in ion peak areas for ethyl acetate, ethanol, and acetic acid yielded by Pinot Noir fruit with diffuse powdery mildew (DPM) caused by *Uncinula necator* relative to healthy fruit, as determined by gas chromatography and mass spectroscopy

Year	Relative increase in ion peak area ^a		
	Ethyl acetate	Ethanol	Acetic acid
2001	258.6	6.5	1.17 × 10 ⁸
2002	41.7	3.9	36.0
2003	13.2	17.5	9.7

^a Ion peak area obtained for DPM fruit divided by that for healthy fruit.

TABLE 1. Effects of an insecticide on infestation of Pinot Noir grape berries by fruit-feeding insects and severity of bunch rot caused by *Botrytis cinerea*^a

Year, category ^b	Powdery mildew (%) ^c		Untreated control		Fenpropathrin treated	
	Incidence	Severity	Infestation (%) ^d	Severity (%) ^e	Infestation (%)	Severity (%)
2001						
Healthy	0.1	0.08	1.3 (0.29)	0.54 (0.10)	0.0 (0)	0.4 (0.09)
DPM	27.3	3.3	38.3 (4.33)	15.9 (0.52)	0.0 (0)	5.9 (1.82)
2002						
Healthy	0.3	0.04	2.7 (0.49)	0.8 (0.11)	0.0 (0)	0.58 (0.14)
DPM	34.8	10.3	49.4 (7.19)	15.6 (1.13)	1.1 (0.67)	4.9 (0.95)

^a Grape berries with or without diffuse powdery mildew (*Uncinula necator*) were sprayed with fenpropathrin at 0.5 g/liter a.i. at veraison and twice more at 10-day intervals in order to reduce infestation of the clusters by fruit-feeding insects. Incidence of insect infestation, incidence and severity of diffuse powdery mildew, and the severity of bunch rot was recorded at harvest in all treatments.

^b “Healthy” fruit were rigorously treated with fungicides to prevent development of powdery mildew. The “diffuse powdery mildew” (DPM) treatment likewise was treated with fungicides, but was inoculated with a conidial suspension of *U. necator* 3 weeks after the completion of bloom and midway between two fungicide applications.

^c Incidence and severity of diffuse infection was estimated at harvest by examining clusters at ×25 magnification with oblique illumination. Incidence was the percentage of berries within a cluster that bore diffuse powdery mildew colonies. Severity was estimated from the percentage of berry surface colonized on 10 berries per sampled cluster.

^d Insect infestation: mean incidence and standard error (in parentheses) of insect infestation was recorded at harvest on 30 clusters in each of three replicates in all treatments.

^e *Botrytis* severity: mean severity and standard error (in parentheses) of bunch rot was recorded at harvest on 30 clusters in each of three replicates in all treatments.

gicide applications at a specific stage of crop development (i.e., ≈ 3 weeks after bloom). In a more natural setting, diffuse powdery mildew might become established through two principle avenues: first, if fungicidal protection lapses for any reason immediately before the fruit acquire a high level of ontogenic resistance at 3 to 4 weeks post bloom; and, second, growth of earlier-established colonies on the fruit might be halted temporarily by the fungi-

Fig. 6. Microflora associated with powdery mildew-free berries of *Vitis vinifera* 'Pinot Noir' (Healthy) versus those supporting diffuse powdery mildew colonies (DPM) in **A**, Geneva or **B**, Lodi in 2000 and in Lodi in **C**, 2001 and **D**, 2002. Within years at each site, vines were subjected to the same fungicide spray program, but clusters in the DPM treatment were inoculated with conidia of *Uncinula necator* midway between two fungicide sprays just before berries became immune to powdery mildew infection (≈ 3 weeks post bloom). Fruit were harvested ripe, and three replicate lots of 100 berries per treatment were washed and the rinsate plated on four semiselective agar media. Heights of bar segments indicate the total number of CFU per 100- μ l sample of the following microorganisms per medium: YMA (yeast medium agar) = all yeasts; YMC (YMA plus cycloheximide) = *Brettanomyces* and *Dekkera* yeasts; LYS (lysine agar medium) = non-*Saccharomyces* yeasts, presumably *Kloeckera*; and MLB (malolactic broth agar with cycloheximide) = lactic acid bacteria. Error bars indicate one standard error of the mean.

static action of demethylation inhibiting (DMI) fungicides, but then resume before fruit acquire sufficient ontogenic resistance. A related phenomenon has been demonstrated with respect to fungistatic effects of DMI fungicides applied to apple foliage and survival of *Venturia inaequalis* (4,10,28), and potentially could be of more concern where efficacy of DMI fungicides has eroded due to population shifts toward decreased fungicide sensitivity (9,22). Diffuse powdery mildew can be prevented if effective protection of fruit is extended to include that period during which fruit are susceptible only to diffuse powdery mildew (15). Although this slightly more intensive use of fungicides might not be accompanied by a macroscopically visible reduction of powdery mildew on the fruit, our previous studies (12,15) have demonstrated that doing so effectively prevents diffuse powdery mildew and the attendant problems associated with this phase of the disease. The precise delimitation of the period of berry susceptibility is somewhat dependent upon climate (i.e., the period of bloom becomes longer and the period of berry development and, consequently, that of ontogenic resistance development becomes more heterogeneous within a fruit cluster as winter temperatures increase) (20,21). This climate-dependent phenological heterogeneity is a common phenomenon in many deciduous tree fruit, but has received little attention as it relates to the distribution of ontogenic resistance and consequent risk of disease. This is especially relevant to the findings reported herein because, as grapevine bloom becomes more heterogeneous with less winter chilling, so would the potential increase for development of diffuse powdery mildew. Part of our continuing research seeks to better define how climate affects grapevine flowering and, thereby, the period during which fruit are susceptible to powdery mildew and other fungal diseases (20,21).

Fig. 7. Composition of epiphytic yeasts isolated from healthy and diffuse powdery mildew (Diffuse infection) berries in **A**, 2001 and **B**, 2002 identified by polymerase chain reaction sequencing methods. Twenty colonies selected arbitrarily from each of three replicate culture plates of lysine medium were subcultured and subsequently identified, and the percentages of the total microflora for each identified genus was calculated. Bars indicate one standard error of the mean.

TABLE 3. Effects of diffuse powdery mildew (DPM) caused by *Uncinula necator* on juice and wine quality from Pinot Noir grape berries

Year, treatment ^a	PH ^b	Soluble solids (%) ^c	Titrateable acids (g/liter) ^d	Tartrate (g/liter) ^e	Malate (g/liter) ^e	Lactate (g/liter) ^e	Acetate (g/liter) ^e
2000							
Without DPM	3.60	20.6	6.15	0.87	0.17	2.94	0.42
With DPM	3.57	20.3	6.05	0.94	0.22	2.69	0.46
2001							
Without DPM	4.04	22.4	5.07	2.79	>0.10	5.10	0.63
With DPM	3.88	22.7	5.43	2.89	>0.10	4.73	0.62
2002							
Without DPM	3.77	19.3	5.43	0.83	0.13	3.01	0.39
With DPM	3.77	18.9	5.43	0.74	0.19	2.90	0.37

^a Grape berries without DPM were protected from infection by an intensive fungicide program. Berries with DPM were protected by the same fungicide program, but were inoculated with a suspension containing *U. necator* at 10⁵ conidia/ml midway between two fungicide applications, ≈3 weeks post-bloom, and developed disease on at least 25% of all berries by the time of harvest.

^b pH of wine after malolactic fermentation, before adjustment of titrateable acids. Within-year differences are not significant at $P = 0.05$ according to Student's *t* test.

^c Soluble solids (primarily sugars) in fresh juice at the time of harvest.

^d Titrateable acid level of wine after malolactic fermentation, before adjustments of acidity.

^e Tartrate, malate, lactate, or acetate levels in wine after malolactic fermentation, before adjustment of titrateable acids.

Fig. 8. Percentage of individuals that applied one or more negative descriptors to wines prepared from Pinot Noir grape berries harvested in 2000, 2001, or 2002 that bore trace levels (incidence = 1 to 4%) of diffuse powdery mildew (Healthy) versus berries bearing numerous (incidence = 27 to 58%) diffuse powdery mildew colonies (DPM). Wines were evaluated 18 months after bottling by 8 to 11 experienced tasters in a double-blind comparison in which each of three replicates per treatment was sampled twice. Negative descriptors of taste or odor that were perceived by an individual in both the first and second sampling of a replicate were compiled and the percentage of individuals on a panel that applied one or more negative attributes to a replicate was recorded. Negative descriptors were: "bretty, adhesive tape, spoiled fruit, Band-aid, manure, barnyard, mousy, burlap, rotted vegetables, musty, dirty, pondwater, algal, stinky, spoilage, sweaty, burnt plastic, aldehyde, urine, bitter, off-aroma, oxidized, unpleasant, and foul." Bars indicate one standard error of the mean.

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