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Increase of Fumonisin B_2 and Ochratoxin A Production by Black *Aspergillus* Species and Oxidative Stress in Grape Berries Damaged by Powdery Mildew

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ABSTRACT

Powdery mildew (PM), caused by the fungus *Erysiphe necator,* is one of the most widespread fungal disease of grape and may cause extensive openings on the berry surface during the infection. We evaluated the effect of damage caused by PM in grape berries on the growth of and mycotoxin production by *Aspergillus* and on the oxidative stress in infected berries. Berries of *Vitis vinifera* L. cv. Negroamaro with sound skin (SS) and those naturally infected by PM were surface sterilized and inoculated with either fumonisin B_2 (FB_2)–producing strains of *Aspergillus niger* or ochratoxin A (OTA)–producing strains of *Aspergillus carbonarius* and incubated at 20 and 30°C. The PM berries were significantly more susceptible to both *Aspergillus* colonization (5 to 15 times more susceptible) and OTA and FB_2 contamination (2 to 9 times more susceptible) than were SS berries. The highest toxin concentration was detected in inoculated PM berries both for OTA (9 ng/g) at 20°C and for FB_2 (687 ng/g) at 30°C. In inoculated SS and PM berries, although malondialdehyde and hydrogen peroxide concentrations did not increase, the two black *Aspergillus* species caused a significant decrease in ascorbate content, thus inducing a pro-oxidant effect. These results indicate that grape berries affected by PM are more susceptible to black *Aspergillus* growth and to production and/or accumulation of FB_2 and OTA. Thus, preventive control of *E. necator* on grape berries could reduce the mycotoxicological risk from black *Aspergillus* infection.

Powdery mildew (PM), caused by *Erysiphe necator,* is one of the most widespread fungal diseases of *Vitis vinifera* and infects all green tissue on the grapevine, including young berries. *E. necator* is an obligate parasite of *Vitis* species that was introduced into Europe and, eventually, all other wine-producing regions of the world. A review of phenotypic and phylogenetic features characterizing this taxon has recently been published *(6)*. PM infection may reduce grape yield and may result in quality changes in the resulting musts and wines *(7, 12)*.

Aspergillus bunch rot is a fungal disease that affects preharvest grapes and is caused by a complex of *Aspergillus* species in section *Nigri,* including *A. carbonarius, A. niger, A. tubingensis,* and *A. uvarum (32, 38)*. The importance of bunch rot escalated markedly when ochratoxin A (OTA) and fumonisins were found as contaminants in grapes and grape-derived products *(21, 24, 36)*. The effects of OTA and fumonisins on cell function have been studied in animal cells in an effort to understand the toxicity of these mycotoxins. OTA is nephrotoxic, immunotoxic, teratogenic, and mutagenic in several species of animals and can cause kidney and liver tumors in mice and rats *(16)*. OTA enhances NADPH-dependent lipid peroxidation in the liver and kidney *(29)* and promotes hydroxyl radical production *(15)*. In plant systems, OTA induced cell death and changes in oxidative metabolism of *Arabidopsis thaliana (31)*. Fumonisins have been extensively studied as mycotoxins with cancer-promoting activity *(13)* and have been associated with a number of animal and human diseases *(17)*. Fumonisin B_2 (FB_2) is more cytotoxic than are other fumonisins *(14)*.

During plant-microbe interactions, a general response of plants to pathogen attack is the production of reactive oxygen species such as superoxide anions, hydrogen peroxide (H_2O_2), and hydroxyl radicals, which at high concentrations may cause lipid peroxidation, membrane deterioration, and protein degradation *(5)*. Efficient antioxidant activity is essential to maintain a low concentration of reactive oxygen species *(18),* mediating the systemic activation of defense gene expression in response to pathogens *(22)*. Ascorbate is an important antioxidant and a free radical scavenger and is indispensable for the regeneration of vitamin E, which prevents membrane lipid peroxidation and acts synergistically with other biological antioxidants such as glutathione *(4)*.

Although the diseases induced by *E. necator* and black *Aspergillus* species are well studied, little information is

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available on their effects. We conducted this study to assess whether the openings caused on the grape berry surface by *E. necator* may affect colonization by *A. carbonarius* and *A. nigri* and accumulation of OTA and FB_2. By monitoring the concentrations of ascorbate and H_2O_2 and the degree of membrane lipid peroxidation in the infected berries after black *Aspergillus* colonization, we estimated the level of oxidative stress.

MATERIALS AND METHODS

Description of trials. Two trials were conducted in vitro in 2009 with grape berries from the wine grape cultivar Negroamaro, which were collected in a vineyard in Apulia, southern Italy. During the ripening stages (about 20 days before the harvest date), 20 healthy grape bunches and 20 grape bunches naturally infected with *E. necator* were randomly collected. The bunch samples were placed in sterile plastic bags, cooled to 4°C, and processed for the trials within 24 h. For the trials, two categories of grape berries were used: (i) berries with sound skin (SS) and (ii) berries naturally damaged by PM (i.e., homogeneously diseased berries with more than 20% of the skin split open). A total of 48 groups, each consisting of 11 berries (sufficient for chemical analysis), were prepared: 24 groups of SS berries were obtained by randomly detaching berries from the healthy bunches that were homogeneous in size and color, with no visible damage to their skin and no visible fungal growth, and 24 groups of PM berries were created by detaching berries from the bunches infected by PM. All the berries were surface disinfected with 20 g/liter sodium hypochlorite for 2 min and rinsed twice with distilled water. Each trial was conducted according to a factorial design with two factors in triplicate. Factor 1 was berry status: SS not inoculated (SS control), SS inoculated, damaged PM not inoculated (PM control), and damaged PM inoculated. Factor 2 was temperature: 20 and 30°C.

A mixture of five FB_2-producing strains of *A. niger* (Istituto Tossine e Micotossine [ITEM] strains 10557, 10584, 10589, 10612, and 10615) isolated from grapes collected in Apulian vineyards was used as the inoculum in trial 1. The isolates were previously identified by β-tubulin gene sequencing *(20)* and tested in vitro for their ability to produce FB_2 *(8)*.

In trial 2, a mixture of five ochratoxigenic strains of *A. carbonarius* (ITEM strains 5006, 5012, 7444, 7475, and 7488) isolated from grapes collected in various Italian vineyards was used as the inoculum. The isolates were previously identified by molecular methods *(32)* and tested in vitro for their ability to produce OTA *(1)*. All the isolates were obtained from the culture collection of the Italian National Research Council, Institute of Sciences of Food Production (Bari, Italy).

Inocula were prepared by growing the strains in petri dishes with potato dextrose agar for 7 days at 25°C. After incubation, each dish was washed with sterile distilled water, and the volume adjusted to give 10^7 conidia per ml. In each trial, a composite suspension was prepared by mixing 100 ml of suspension from each strain. Each group of grape berries in the SS- and PM-inoculated groups was dipped into the *Aspergillus* conidial suspension for 5 min. The berries of each replicate were then put into a humidity chamber (a sterile 12-well microplate with lid). One berry was placed in each well, and one well in each plate contained a piece of paper moistened with 8 ml of sterile water. The microplate loaded with berries was sealed with Parafilm M (Pechiney Plastic Packaging Co., Chicago, IL) and incubated for 7 days at 20 or 30°C. After incubation, the berries of each replicate were homogenized with an Ultra Turrax homogenizer (T 25 digital, IKA, Wilmington, NC) at 15,000 rpm for 90 s. The slurry samples were frozen at −20°C and then processed to determine the levels of black *Aspergillus* and the OTA and FB_2 concentrations.

Isolation and quantification of contamination by *Aspergillus* section *Nigri*. *Aspergillus* populations in the slurry obtained from each replicate were determined according to Cozzi et al. *(9)*. The slurry was used to make serial 10-fold dilutions in water. From each dilution, 100 µl was spread in triplicate on the surface of dichloran rose Bengal chloramphenicol agar (Oxoid, Basingstoke, UK) in petri dishes (90-mm diameter). All dishes were incubated in the dark for 4 days at 25°C. The black *Aspergillus* colonies were identified based on morphology *(33)*. The number of *Aspergillus* colonies was counted at ×40 magnification directly on the plate. The black *Aspergillus* population was determined as CFU per gram of slurry, and the values were log transformed for statistical analysis.

Determination of OTA in grape berries. OTA was measured as previously described by Solfrizzo et al. *(35)*. Five grams of grape purée was extracted with 26 ml of high-performance liquid chromatography (HPLC) grade acetonitrile-methanol-water (90:90:80, vol/vol/vol; Mallinckrodt Baker, Deventer, The Netherlands). Ultrapure water was produced with a Milli-Q system (Millipore, Bedford, MA) by shaking for 60 min. After filtration through filter paper (no. 4; Whatman, Maidstone, UK), 6 ml of filtrate was diluted with 44 ml of an aqueous solution containing 1% polyethylene glycol 8000 and 5% $NaHCO_3$, and this solution was filtered through a glass microfiber filter (Whatman GF/A). A 10-ml volume of diluted extract (equivalent to 0.2 g of sample) was cleaned in an OchraTest immunoaffinity column (Vicam, Watertown, MA) at a flow rate of 1 drop per s. The column was washed with 10 ml of a solution containing 2.5% NaCl and 0.5% $NaHCO_3$ followed by 10 ml of distilled water. OTA was eluted with 2 ml of methanol and collected in a silanized vial (Supelco, Sigma-Aldrich, St. Louis, MO). The eluted extract was evaporated under a nitrogen stream at ca. 50°C and reconstituted with 500 µl of the HPLC mobile phase.

A 100-µl volume of reconstituted extract was injected into the HPLC apparatus (technology series 1100, Agilent, Santa Clara, CA) with a full loop injection system. The fluorometric detector was set at wavelengths of 340 nm (excitation) and 460 nm (emission). The analytical column was a Zorbax SB-C18 (4.6 by 150 mm by 5 µm; Agilent) with a guard column inlet filter (0.5 µm by 3 mm in diameter; Rheodyne Inc., Rohnert Park, CA). The mobile phase consisted of a mixture of acetonitrile–water–glacial acetic acid (99:99:2, vol/vol/vol) at a flow rate of 1 ml/min.

OTA was quantified by measuring peak areas at the retention time of OTA standard solutions (Sigma-Aldrich, Milan, Italy) and comparing these areas with the relevant calibration curve at 0.05 to 100 ng/ml. Mean OTA recovery from grapes spiked at 1, 10, and 50 ng/g was 78%, with a relative standard deviation of about 6%. The limit of quantification for OTA was 0.1 ng/g.

Determination of FB_2 in grape berries. FB_2 was analyzed according to the method of De Girolamo et al. *(10)*. Ten grams of ground grape berries (purée) was extracted with 20 ml of HPLC-grade acetonitrile-methanol (1:1, vol/vol; Mallinckrodt Baker) and 12 ml of ultrapure water (Milli-Q system) by orbital shaking for 60 min. The extract was centrifuged for 10 min at 4,000 × *g* and filtered through filter paper (Whatman no. 4). Ten milliliters of homogenized extract was diluted with 40 ml of 0.1 M phosphate-buffered saline (PBS) and filtered through a glass microfiber filter (Whatman GF/A). A 10-ml volume (equivalent to 0.5 g of sample) was passed through the FumoniTest immunoaffinity column

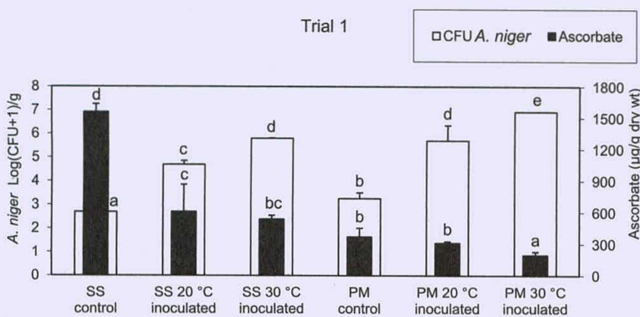

FIGURE 1. *Ascorbate concentrations and populations (log [CFU + 1]/g) of* A. niger *in Negroamaro grape berry samples in trial 1. Values with the same letter are not significantly different at* P < *0.05 according to the Duncan test.*

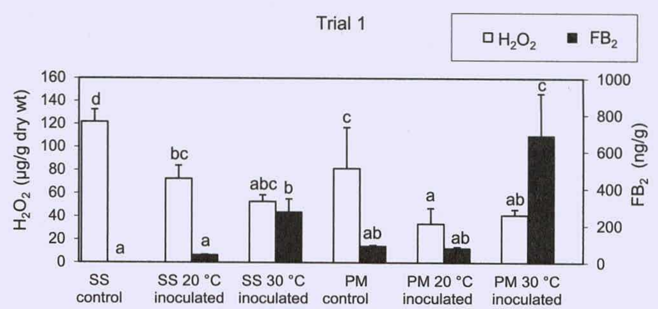

FIGURE 2. *Fumonisin B_2 and H_2O_2 concentrations detected in Negroamaro grape berry samples in trial 1. Values with the same letter are not significantly different at* P < *0.05 according to the Duncan test.*

(Vicam). The column was washed with 10 ml of PBS, and FB_2 was eluted with 2 ml of methanol and 2 ml of water. The eluates were evaporated to dryness under a stream of nitrogen at 50°C. The residues were dissolved with 500 µl of acetonitrile-water (30:70, vol/vol), and 110 µl of the extract was derivatized with 110 µl of *o*-phthaldialdehyde (OPA solution, Sigma-Aldrich) using the HPLC autosampler (Varian Inc., Palo Alto, CA). This solution was mixed for 30 s, and 50 µl (0.025 g of sample) was injected by full loop 3 min after adding the OPA reagent for fumonisin analysis.

The analytical column was a Symmetry Shield RP18 (15 cm by 4.6 mm, 5 µm; Waters, Milford, MA) with a guard column inlet filter (0.5 µm by 3 mm diameter; Rheodyne), and the mobile phase, consisting of a binary gradient, was applied as follows. The initial composition of the mobile phase was 60% solvent A (acetonitrile–water–acetic acid, 30:69:1, vol/vol/vol) and 40% solvent B (acetonitrile–water–acetic acid, 60:39:1, vol/vol/vol) and was kept constant for 5 min, then solvent B was linearly increased to 88% over 21 min and kept constant for 4 min. To clean the column, the amount of acetonitrile was increased to 100% and kept constant for 4 min. The column was set with a thermostat at 30°C. The flow rate of the mobile phase was 1 ml/min. The fluorometric detector was set at wavelengths of 335 nm (excitation) and 440 nm (emission).

FB_2 (Sigma-Aldrich, Milan, Italy) was quantified by measuring peak areas and comparing them with a calibration curve of 8 to 500 ng/ml obtained with standard solutions. Mean FB_2 recovery from grapes spiked at 100, 500, and 1,000 ng/g was 82%, with a relative standard deviation of about 10%. Quantification limit for fumonisins was 10 ng/g based on a signal-to-noise ratio of 10:1.

Lipid peroxidation, H_2O_2, and ascorbate measurements. For assays of ascorbate and H_2O_2 and for lipid peroxidation analysis, grape slurry samples of each replicate were lyophilized and homogenized with appropriate extraction buffer.

For lipid peroxidation, 2 g of lyophilized berries was homogenized with 4 ml of 0.1% (vol/vol) trichloroacetic acid. The homogenate was filtered through three layers of cheesecloth and centrifuged at 15,000 × *g* for 20 min. The degree of lipid peroxidation was measured in terms of malondialdehyde concentration as described by Paciolla et al. *(30)*.

For analysis, 2 g of each sample was homogenized in 6 ml of 100 mM sodium phosphate buffer, pH 6.8. The homogenate was filtered through three layers of cheesecloth and centrifuged at 18,000 × *g* for 20 min, and the H_2O_2 concentration of the supernatant was measured according as described by Lee and Lee *(19)*.

For determination of ascorbate concentration, 2 g of each lyophilized sample was ground with three volumes of cold 5% (wt/vol) metaphosphoric acid in a porcelain mortar. The homogenate was filtered and centrifuged for 15 min at 20,000 × *g*, and the supernatant was collected for analysis of ascorbate concentration according to the method of Zhang and Kirkham *(40)*.

Statistical analysis. All experiments were performed in triplicate and repeated at least three times. An analysis of variance was applied to the data, and means were compared using the Duncan test ($P < 0.05$) to identify significant differences. Factor analysis was also performed for factors 1 (berry status), 2 (temperature), and 3 (black *Aspergillus* group). Regression analysis of black *Aspergillus* species population versus H_2O_2 concentration was performed for berry samples contaminated with black *Aspergillus* ($n = 30$). All statistical analyses were performed with the STATISTICA version 6.0 software package for Windows (Stat-Soft, Tulsa, OK).

RESULTS

Infection of grape berries by FB_2-producing strains. The degree of colonization by *A. niger* and the concentration of FB_2 in inoculated PM and SS berries at the two incubation temperatures are shown in Figures 1 and 2, respectively. The inoculated SS and PM berries were more colonized and contaminated than were the respective controls. In trial 1, the inoculated PM berries incubated at 30°C were the most heavily colonized by *A. niger* ($P < 0.05$) (Fig. 1) and contained the highest concentrations of FB_2, an average of 687 ng/g ($P < 0.05$) (Fig. 2). The PM and SS berries inoculated at 20°C had lower levels of *A. niger* colonization (more than 10 times lower) compared with the same berries incubated at 30°C ($P < 0.05$) (Fig. 1). SS and PM berries inoculated at 20°C had average FB_2 values of 42 and 78 ng/g, respectively, more than six times lower than those of the SS and PM berries incubated at 30°C ($P < 0.05$) (Fig. 2). In trial 1, a positive and significant correlation ($r = 0.71$, $P < 0.01$) was found between *A. niger* levels and FB_2 concentrations.

Infection of grape berries by OTA-producing strains. The degree of colonization of *A. carbonarius* and the concentration of OTA in inoculated PM and SS berries at the two incubation temperatures are shown in Figures 3 and 4, respectively. The inoculated SS and PM berries were more colonized and contaminated than were the respective controls. In trial 2, the PM-inoculated berries incubated

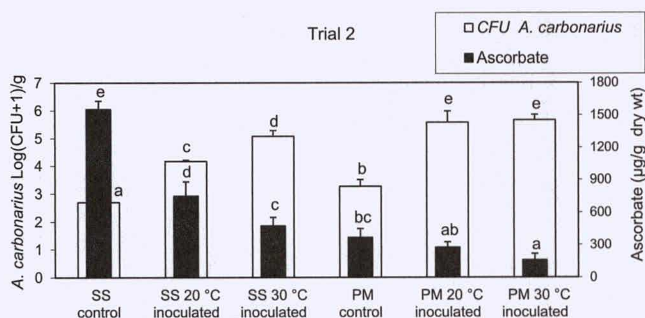

FIGURE 3. *Ascorbate concentrations and populations (log [CFU + 1]/g) of* A. carbonarius *in Negroamaro grape berry samples in trial 2. Values with the same letter are not significantly different at* P *< 0.05 according to the Duncan test.*

at both 20 and 30°C were the most heavily colonized by *A. carbonarius* ($P < 0.05$). No significant difference ($P < 0.05$) was observed between the PM-inoculated berries incubated at 20 and 30°C with respect to *A. carbonarius* colonization (Fig. 3). At 30°C, the inoculated PM berries were about five times more colonized by *A. carbonarius* (Fig. 3) and two times more contaminated by OTA (4.7 versus 2.16 ng/g) (Fig. 4) than were inoculated SS berries. At 20°C, the inoculated PM berries were about 15 times more heavily colonized by *A. carbonarius* and 9 times more contaminated by OTA (9 versus 0 ng/g) compared with the SS-inoculated berries at the same temperature ($P < 0.05$) (Fig. 4). OTA was not detected in the undamaged (SS) inoculated berries at 20°C.

After inoculation, at both temperatures the PM damaged berries were on average about eight times more heavily colonized by black *Aspergillus* than were inoculated SS berries (Fig. 5). *A. niger* strains colonized both types of berries significantly more heavily ($P = 0.01$) than did *A. carbonarius* strains (Fig. 5). In trial 2, a positive and significant correlation ($r = 0.74$, $P < 0.01$) also was found between *A. carbonarius* levels and OTA concentration.

Ascorbate, H_2O_2, and lipid peroxidation. In the PM control berries of the trials 1 and 2, the ascorbate concentration was about 2.8 times lower than that in the SS control berries (Figs. 1 and 3). A decrease in ascorbate concentration also occurred in all samples inoculated with *A. niger* (Fig. 1) or *A. carbonarius* (Fig. 3) compared with the SS and PM controls. However, the decrease in ascorbate

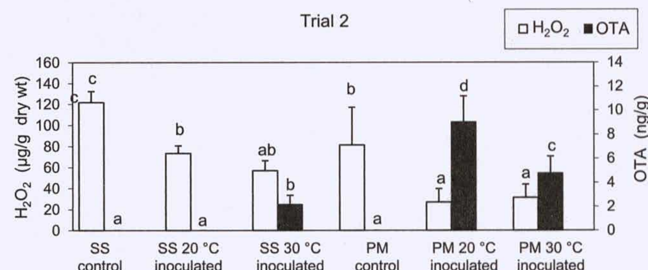

FIGURE 4. *Ochratoxin A and H_2O_2 concentrations detected in Negroamaro grape berry samples in trial 2. Values with the same letter are not significantly different at* P *< 0.05 according to the Duncan test.*

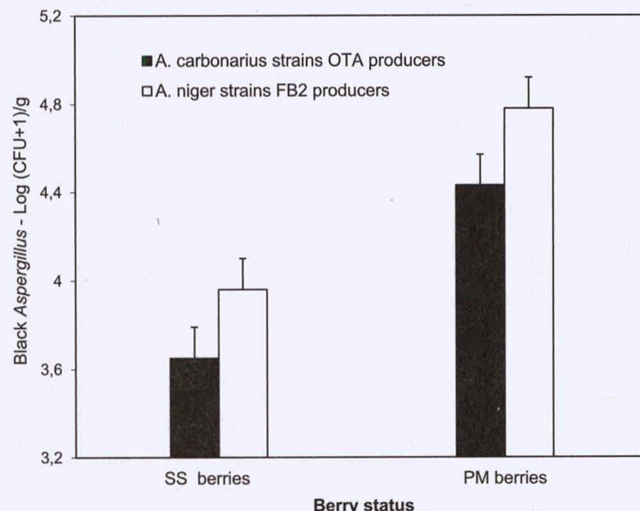

FIGURE 5. *Interaction between black* Aspergillus *(*A. carbonarius *and* A. niger *mycotoxin-producing strains) and grape berry status (SS or PM),* P = *0.01. Vertical bars indicate 95% confidence intervals of means.*

was greater in the inoculated SS berries than in the inoculated PM berries and was greater at 30°C than at 20°C.

The H_2O_2 concentration was lower in the berries infected by *E. necator* (PM control) than in the SS controls (Fig. 2). The H_2O_2 decrease also occurred in all samples inoculated with *A. niger* or *A. carbonarius* and was more marked in inoculated PM berries than in inoculated SS berries (Figs. 2 and 4). No significant difference between berries incubated at 20°C and those incubated at 30°C was observed.

The relationship between colonization of by *Aspergillus* species and H_2O_2 concentration detected in the PM and SS berries at both temperatures also was analyzed (Fig. 6), and a strong negative correlation was found ($r = -0.7995$).

When lipid peroxidation (measured in terms of malondialdehyde concentration) was analyzed, no significant differences were detected among the berry groups (data not shown).

DISCUSSION

In the two trials, at the same incubation temperatures the highest levels of black *Aspergillus* species were observed in the PM grape berries. This result indicates that *A. niger* and *A. carbonarius* berry rots are enhanced by preexisting *E. necator* infection, which can easily crack the berry skin. The interspecific interactions of these opportunistic *Aspergillus* species and *E. necator* were not evaluated in this in vitro study, although the results suggest that severe PM infection leads to splitting of the berries, which may predispose the berries to attacks by various fungi including those of the *A. niger* group. Both berry types used in these trials were more heavily colonized by *A. niger* strains than by *A. carbonarius* strains. This difference may be due to many factors, including a lower growth rate of *A. carbonarius* than of *A. niger* during the incubation time at the tested temperatures (20 and 30°C). Our results are similar to those of Bellí et al. *(3)*, which were obtained on a synthetic nutrient medium similar in composition to grape berries.

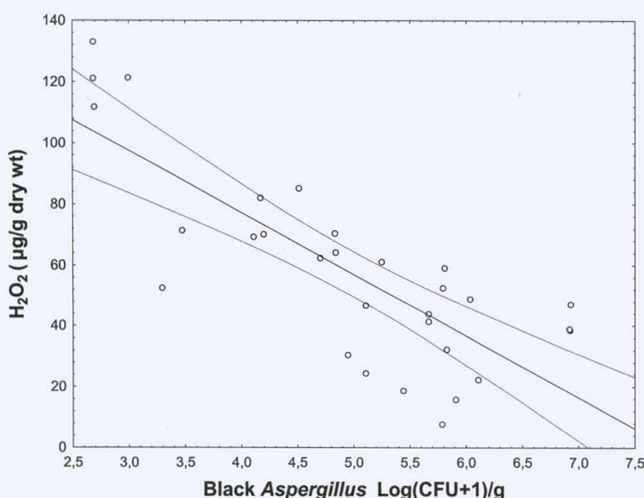

FIGURE 6. *Regression analysis of fungal population (CFU) versus H_2O_2 concentration showing the correlation between black* Aspergillus *levels in berries and their H_2O_2 concentrations (y = 158.2881 − 20.2616 × x; r = −0.79) with 95% confidence intervals.*

In the two trials, a similar positive correlation between the levels of black *Aspergillus* species and mycotoxin concentration was found. However, in trial 1, the concentration of FB_2 detected in the inoculated berries was always greater than that of OTA in the inoculated berries in trial 2. The maximum concentrations of OTA and FB_2 were recorded at 20 and 30°C, respectively. This trend indicates that the optimal temperature for fumonisin synthesis is higher than that for OTA, as reported by other authors *(2, 25)*.

An increase in reactive oxygen species occurs during plant-pathogen interactions. However, the strong negative correlation between berry colonization by black *Aspergillus* and H_2O_2 concentration and the lower H_2O_2 concentrations observed in the berries infected by different pathogens (*E. necator, E. necator* plus *A. niger,* and *E. necator* plus *A. carbonarius*) indicate a poor defensive plant response. H_2O_2 is a substrate for apoplastic peroxidases in the polymerization process of monolignols in lignin formation, and a peroxidase isoenzyme has been localized in xylem vessels of grape berries *(26)*. Lower H_2O_2 concentrations could lead to reduced lignification and weaken the physical defense barrier against pathogens and/or favor the diffusion of mycotoxins produced by pathogens.

The decrease in H_2O_2 concentration in inoculated SS and PM berries compared with the controls is probably responsible for the unchanged lipid peroxidation in all inoculated samples. This oxidative process, which causes a peroxidative breakdown of unsaturated fatty acids and consequent swelling of cell membranes, which triggers cell death, is activated by high levels of reactive oxygen species such as H_2O_2. However, a different trend of increased malondialdehyde has been observed in peach fruit inoculated with *Penicillium expansum* at 20°C *(39)*.

Several authors have reported that during the latter stages of disease, several fungal toxins can elicit defense responses in plants that are similar to well-known pathogen-induced responses. In all of these studies, higher peroxidase, chitinase, and glucanase activities *(11)* or higher ascorbate concentration and peroxidase activity *(30)* were found. The effectiveness of ascorbate could thus be one of the factors controlling fungal colonization and therefore could be correlated with induced resistance of the host. However, in diseased grape berries, the presence of OTA and FB_2 mycotoxins did not seem to elicit the fruit defenses, as indicated by significantly decreased ascorbate concentrations. In contrast, the lower ascorbate concentration depotentiates the antioxidant defenses, triggering a pro-oxidant effect in diseased berries. A decline in ascorbic acid concentration over time when compared with control fruits has been reported in guava infected by *A. niger (34)* and tomato fruits inoculated with *Fusarium equiseti, Fusarium chlamydosporium, Geotrichum candidum, Acremonium recifei, Aspergillus flavus,* and *A. niger (28)*. Thus, the decrease in ascorbate concentration observed in the PM control and all inoculated samples compared with the SS control could be due to fungal colonization. The lower ascorbate concentration in PM berries inoculated with *Aspergillus* species compared with the PM control berries suggests that the co-occurrence of these fungi reduces the defense systems of plant cells. Ascorbate is a highly specific electron donor for prolyl hydroxylase and is necessary for the hydroxylation reaction of hydroxyproline-rich glycoproteins (HRGPs) *(37)*. Wall proteins such as HRGPs increase in the cell walls as the berries develop *(27)*. Thus, a low ascorbate concentration could in part modify the interaction of HRGPs with other wall components and affect the assembly and strength of the cell wall in berries. After inoculation of berries with both *Aspergillus* species, we found a far greater decrease in ascorbate concentration at 30°C than at 20°C and the level of fungal growth was correspondingly higher. In maize grain, fungal interactions may act as an additional control factor leading to a decrease in colonization by other pathogens and lower levels of mycotoxins *(23)*. In the present study, the berries infected with *E. necator* were more susceptible to colonization by black *Aspergillus* species and to consequent increases in mycotoxins. The higher levels of black *Aspergillus* species and higher concentrations of mycotoxins detected in the berries damaged by PM highlight the importance of controlling *E. necator* for maintaining the safety of grape-derived products.

In conclusion, PM infection may predispose grape berries to enhanced mycotoxicological risk through infection by black *Aspergillus* species.

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