Resveratrol Oxidation in *Botrytis cinerea* Conidia

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Accepted for publication 6 February 1998.

ABSTRACT

Adrian, M., Rajaei, H., Jeandet, P., Veneau, J., and Bessis, R. 1998. Resveratrol oxidation in *Botrytis cinerea* conidia. Phytopathology 88:472-476.

Observations using light microscopy showed that approximately 30% of *Botrytis cinerea* conidia treated with semi-lethal concentrations (i.e., 60 µg/ml) of the grapevine phytoalexin resveratrol possessed intracellular brown coloration. This coloration was never observed in the absence of resveratrol or in conidia treated with resveratrol together with sulfur dioxide (antioxidant compound) or sodium diethyldithiocarbamate (inhibitor of laccase action), suggesting that discoloration resulted from the laccase-mediated oxidation of resveratrol. Further studies using transmission electron microscopy enabled the observation of particular intravacuolar spherical vesicles and of granular material deposits along the tonoplast. These observations are likely to be related to the oxidation of resveratrol by an intracellular laccase-like stilbene oxidase of *B. cinerea.*

The production of phytoalexins is considered to be part of the general defense mechanism of grapevines (6,13,17,27,28). In *Vitis* spp., such a response includes the formation of a range of biosynthetically related di- and oligomers of a simple stilbene, resveratrol (*trans*-3,5,4′-trihydroxystilbene) (6,17,21,24,26,31), together with the formation of a dimethylated stilbene, pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene) (25). These compounds are typically accumulated in leaves and berries in response to stresses that include fungal infection, UV irradiation, and induction by chemicals (1,5,24,26,31).

Resveratrol is quantitatively the major component in grapevine phytoalexin response. Its accumulation is correlated with resistance to various fungal organisms (3,6,20,21,24,42) and, at physiological concentrations, is fungitoxic against *Botrytis cinerea*, the causal agent for gray mold (2). However, previous results have shown that *B. cinerea* secretes a laccase-like stilbene oxidase capable of oxidizing resveratrol (16,22,40,41).

This work presents particular cytological and ultrastructural characteristics of *B. cinerea* conidia after exposure to resveratrol modified by laccase activity. These characteristics, which have never been described, are of particular relevance in the study of the *B. cinerea*-grapevine interaction.

MATERIALS AND METHODS

Culture of *B. cinerea* and preparation of conidial suspensions. The *B. cinerea* strain used for this work was collected at the viticultural and enological experiment station of the University of Bourgogne on mature grape berries of cultivar Pinot noir. Conidia were suspended in water, inoculated on potato dextrose agar (Bio Mérieux Corp., Lyon, France) and incubated at 21°C. The strain was purified by several successive subcultures and the use of streptomycin (200 mg/liter). The single-spore strain was then stored at 4°C in tubes containing potato dextrose agar slants.

For further experimentation, the strain was grown on potato dextrose agar in 86-mm petri dishes at 21°C with a 12-h light photoperiod. Conidia were aseptically harvested from 10-day-old cultures by suction (39) and transferred into 250-ml Erlenmeyer flasks containing 100 ml of a liquid nutrient-rich medium described by Fournioux and Bessis (11) and similar to that of Hoos and Blaich (16) or Ward and Stoessl (45). The conidial suspension was vibrated for 2 to 5 min with a vortex mixer and diluted to obtain a final concentration of about 10^5 conidia per ml. The conidial suspension was then added to sterile 35-mm petri dishes and immediately incubated with resveratrol.

Incubation of resveratrol with the conidial suspension. Prior to incubation, the purity of resveratrol was checked by high-pressure liquid chromatography (HPLC) analysis using a photodiode array detector (1,19) and by gas chromatography (GC) as previously described (18). Resveratrol (Sigma Chemical Co., St. Louis) was added as an ethanolic solution to the nongerminated conidial suspension at 60 µg/ml. The final concentration of ethanol in the culture medium was 4% to ensure solubility of the stilbene (41). Under these conditions, conidial germination and mycelial growth of *B. cinerea* were not inhibited (data not shown). The control contained just 4% absolute ethanol in the conidial suspension. Sodium diethyldithiocarbamate, 10 mM (an inhibitor for laccase) (23,29,30,36,46), or sulfur dioxide, 0.23 mM (an antioxidative compound) (Prolabo, Paris) (7), was eventually added to the resveratrol solution to prevent its oxidation by *B. cinerea* laccase (29). Liquid cultures were protected from light (to avoid photochemical isomerization of the *trans* form of stilbenes to the less active *cis* form) (26), agitated with a reciprocal shaker (120 rpm), and kept at 21°C. There were five replicates per treatment and experiments were carried out 10 times.

Light microscopy. A drop of the conidial suspension was placed on a glass slide for each treatment and examined with a Leitz Laborlux D microscope (Leitz GmbH, Wetzlar, Germany). Staining with "neutral red" (Kuhlmann, Paris) was utilized to examine the vacuolar system.

Electron microscopy. After 48 h of incubation with an ethanolic solution of resveratrol or ethanol (control), *B. cinerea* suspensions were prepared for electron microscopy. Two samples were collected and examined per treatment (resveratrol and control). The suspensions of *B. cinerea* conidia were first passed through a Swinnex 13-mm filtration system (Poly Labo, Paul Block and Cie, Strasbourg, France) using a disk of Whatman paper (No. 1; Whatman

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Publication no. P-1998-0325-02R

International, Ltd., Maidstone, England) for filtration. Conidia were then retained on the filter paper. The following protocol was used to prepare conidial samples. The fungus was washed in 0.1 M of phosphate buffer (pH 7.2) and added with fixing solution (2.5% glutaraldehyde and 0.1 M NaH_2PO_4-Na_2HPO_4, pH 7.2). After 4 h at 4°C, samples were washed six times and left overnight in 0.1 M of phosphate buffer (0.1 M NaH_2PO_4-Na_2HPO_4, pH 7.2) and then placed in citrate-phosphate buffer solutions of decreasing pH: 6.4, 6.0, 5.4, and 5.0. Half of the samples (a control and one treated with resveratrol) were then dipped in a syringaldazine solution (3,5-dimethoxy-4-hydroxy-benzaldehydazine; Sigma-Aldrich Chimie S.A.R.L., L'Isle d'Abeau, France; cytochemical reaction for laccase activity) in citrate buffer for 150 min followed by buffer replacement and overnight soaking. The other half of the samples were placed in the citrate buffer without syringaldazine. All samples were then placed in buffer solutions of increasing pH: 5.0, 5.4, 6.0, 6.4, and 7.2. The Swinnex filtration system was then dismounted and samples were postfixed in phosphate buffer, pH 7.2, containing 1% (wt/vol) OsO_4 for 4 h at 4°C. After careful washing in phosphate buffer and ultra-pure water (to avoid the precipitation of dimethoxypropane [DMP] by phosphate salts), dehydration was carried out in acidified DMP (38,43) for 10 min. DMP allows rapid dehydration and prevents the dissolution of the reaction product that occurs with acetone or absolute ethanol (15). The material was then removed from the filter and transferred to propylene oxide and embedded in Araldite-Epon resin mixture. Silver-gray sections were cut on a Sorvall MT-2B ultramicrotome (Ivan Sorvall, Inc., Newton, CT), mounted on copper grids, and stained in 2.5% uranyl acetate in 50% methanol for 30 min, followed by lead citrate for 5 min. The sections were viewed in an H-600 electron microscope (Hitachi Ltd., Tokyo) at an accelerating voltage of 75 kV with a 30-µm objective aperture.

RESULTS AND DISCUSSION

Light microscopy observations of liquid cultures of *B. cinerea* conidia incubated with a sublethal concentration of resveratrol (60 µg/ml) showed that approximately 30% of the conidia contained an intracellular brown pigmentation after at least 48 h of incubation. This discoloration was either well delimited or occurred throughout

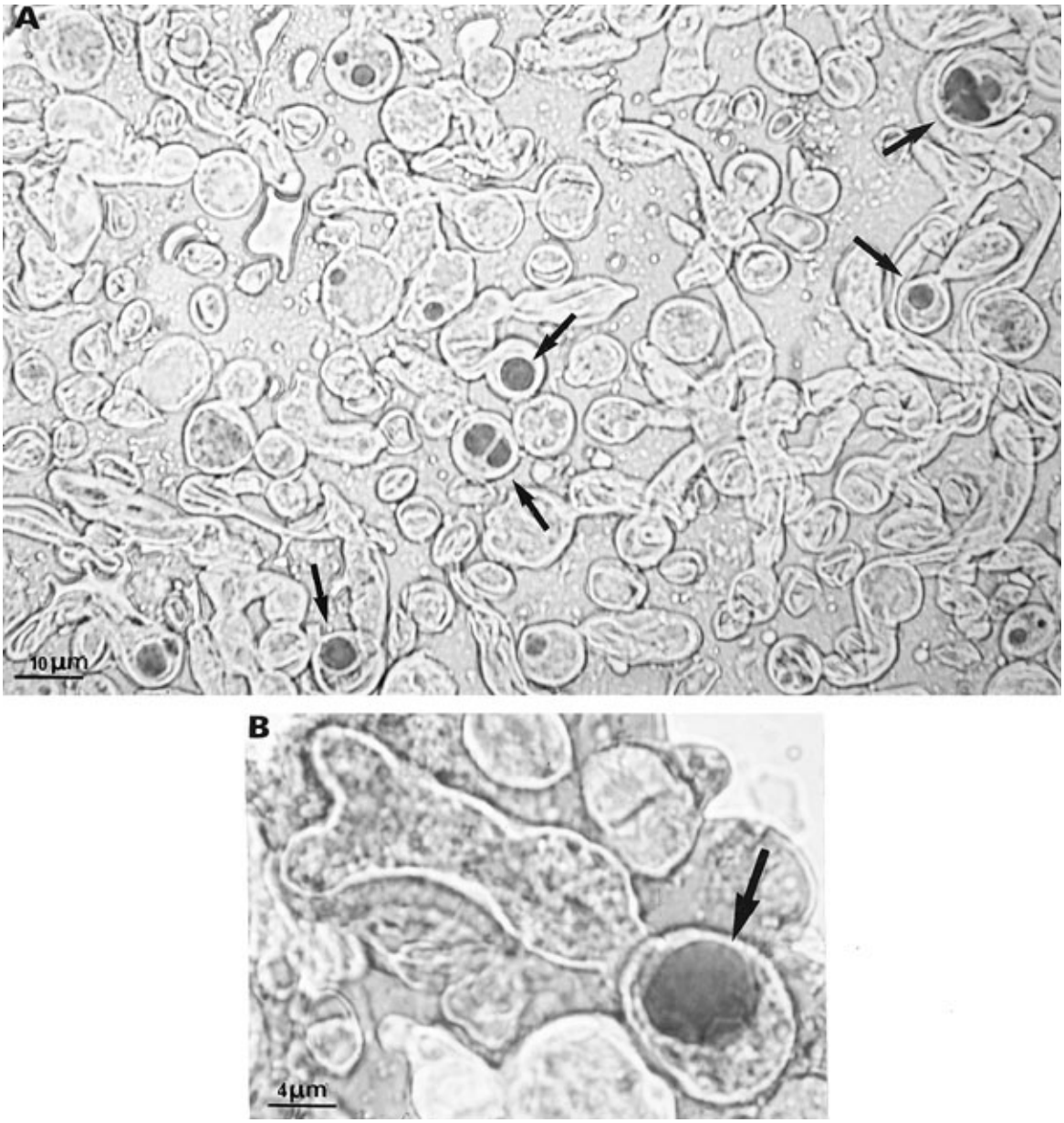

Fig. 1. Light microscopy observations of *Botrytis cinerea* conidia treated with 60 µg/ml of resveratrol for 48 h. Note the occurrence of a brown coloration (arrows) present either **A,** in the entire cytoplasm or **A and B,** is clearly delimited.

the entire conidial cytoplasm (Fig. 1). In contrast, no discoloration was evident in the controls. Brown coloration as described above has been reported in liquid cultures of *B. cinerea* containing hydroxystilbenes (16), but has never been described in fungal cell structures. According to these authors, this discoloration corresponds to an enzymatic oxidation of resveratrol by an extracellular polyphenol oxidase of *B. cinerea* (laccase-like enzyme) secreted in liquid cultures of this fungus. The discoloration observed within the conidia in our system could be attributed to a similar oxidation process. The fact that no discoloration was evident in the controls suggests that resveratrol, like many other phenolics, could act as an inducer of laccase production in *B. cinerea* conidia (32). In the model of Hoos and Blaich (16), disappearance of resveratrol from liquid culture media inoculated with *B. cinerea* was indeed accompanied by a brown pigmentation of the medium that could not be accelerated by the addition of H_2O_2. In our experiments, no coloration was observed in the culture medium containing conidia plus resveratrol, showing that *B. cinerea* conidia do not secrete laccase at this stage.

It is well established that *B. cinerea* produces an extracellular laccase-like polyphenol oxidase capable of oxidizing hydroxystilbenes, namely resveratrol (16,22,40,41). Laccase (*p*-diphenol:oxygen oxido-reductase, EC 1.10.3.2) (32,36), a glycoprotein produced either by plants (23,29,35) or by fungi (30), is capable of oxidizing a wide range of phenolics including mono-, di-, and triphenols (34,36). An extracellular laccase from *B. cinerea* has been purified (8,37). The molecular weight of *B. cinerea* laccase is approximately 60,000 (37), containing at least one copper atom per molecule, and shows unusual properties including a very low isoelectric point (pI 2.5) (9,37,46). In the literature, the heterogeneity of the laccases from *B. cinerea* is clearly demonstrated; their properties mainly depend on the nature of their inducers (32). Our observations concerning the occurrence of a brown coloration in conidia (as a result of laccase-mediated oxidation of resveratrol) are in agreement with those of Mayer and Harel (36), describing the formation of highly colored products, often condensed molecules of high molecular weight, following the action of laccase on phenolic compounds.

We attempted to find out whether the discoloration observed in *B. cinerea* conidia could be attributable to laccase-mediated oxidation of resveratrol by incubating conidia plus resveratrol together with the antioxidative compound sulfur dioxide (7) or with

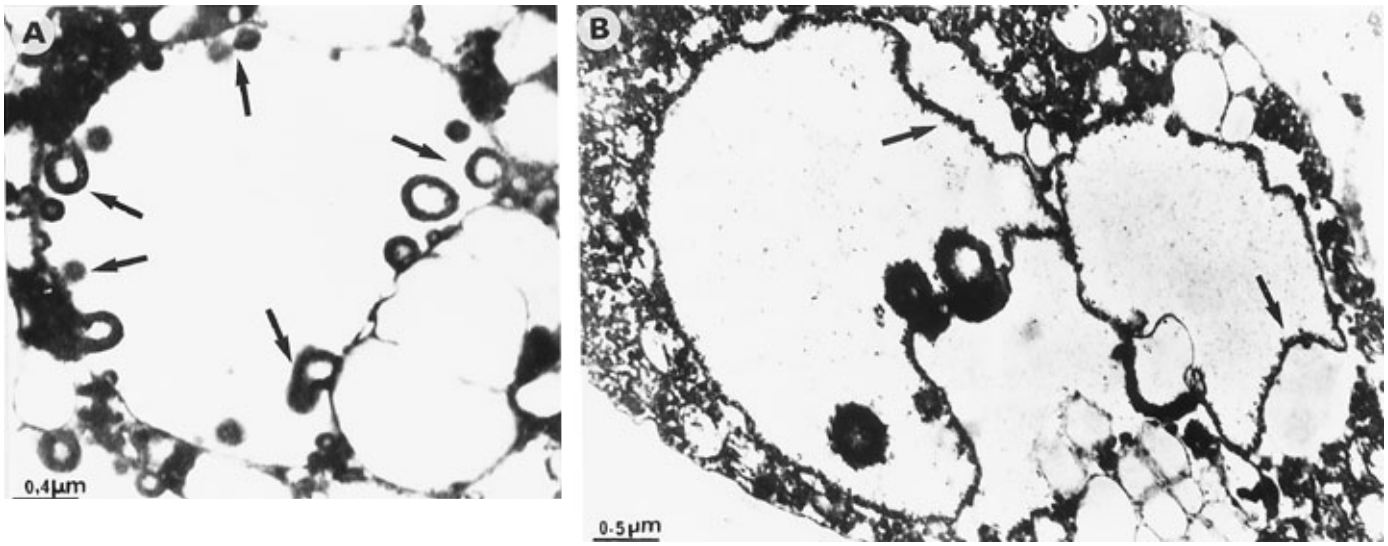

Fig. 2. Transmission electron microscopy of transverse section of *Botrytis cinerea* conidia treated with 60 µg/ml of resveratrol for 48 h. Note the occurrence **A,** of vesicles (arrows) in the vacuoles and **B,** of coarsely granular deposits (arrows) along the tonoplast.

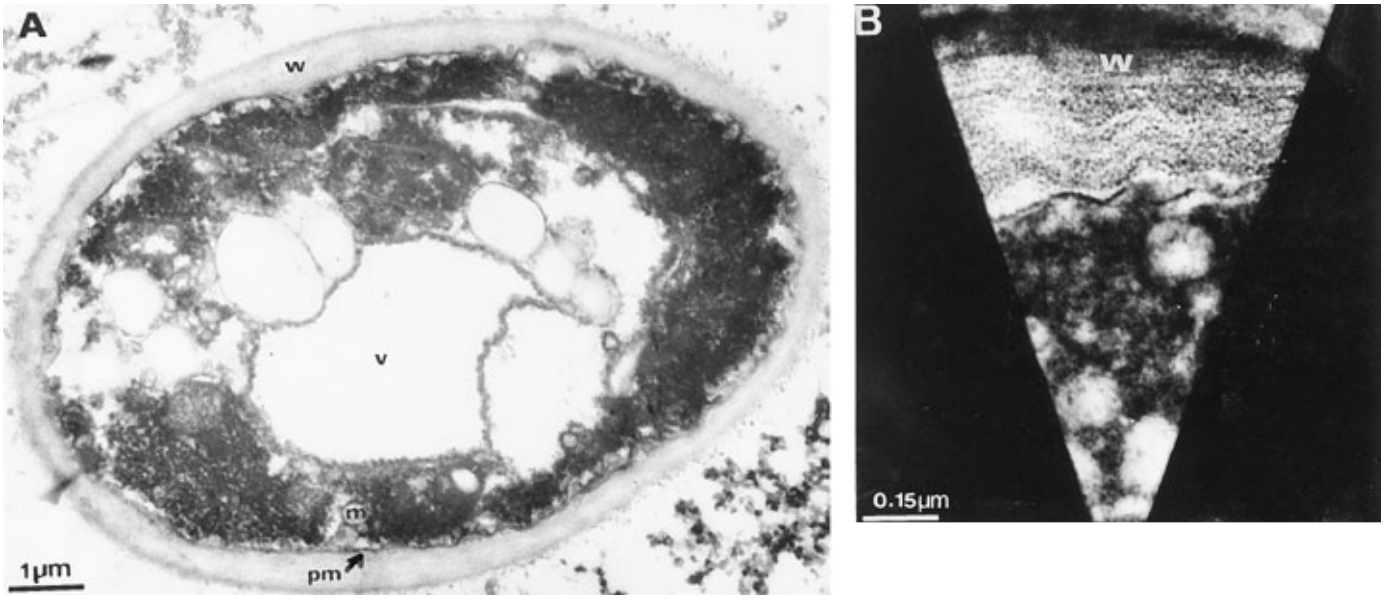

Fig. 3. Cytochemical localization of laccase using syringaldazine as the substrate. Note **A,** the cytoplasmic and **B,** parietal localization of the enzyme, as indicated by electron-dense deposits. Note that laccase is not detected in vacuoles. m= mitochondria, pm = plasma membrane, v = vacuole, and w = wall.

the specific laccase inhibitor sodium diethyldithiocarbamate (23, 29,30,36,46). In both cases, no discoloration was observed in conidia. These data thus suggest that an enzymatic process of resveratrol oxidation, specifically linked to a laccase-like polyphenoloxidase, takes place in the conidia of *B. cinerea.*

This oxidation process (or the accumulation of resveratrol oxidation products) could be located in vacuoles, because the area of the sporeling where the brown coloration was observed is also colored by neutral red, a staining specific for the vacuolar system.

An electron microscopy study showed that the ultrastructure of *B. cinerea* conidia treated with ethanol (control) was the same as that previously described by other authors (4,12,39). However, some of the conidia treated with 60 µg of resveratrol per ml presented different cytological abnormalities that have, to our knowledge, never been described. The tonoplast was often lined with electron-dense material, mainly along the vacuole (Fig. 2B). The brown discoloration observed in light microscopy could thus correspond to this coarse material present along the tonoplast. Spherical vesicles were also observed in the vacuole or along the vacuolar side of the tonoplast. They were either well delimited (by an electron-dense ring different from a membrane or a wall), fully or partly filled with coarsely granular material (in some cases), or even empty (Fig. 2A).

Vesicle size was also not uniform, varying from 0.1 to 0.4 µm. Different vesicular forms could be observed in the same vacuole. Their occurrence was restricted to conidia treated with resveratrol. They differ from the storage bodies described by Buckley et al. (4) and Gull and Trinci (12). The nature and function of these structures still remain unknown. Are they related to the oxidation process of resveratrol by fungal laccase, as they were not observed in the controls? If so, detoxification of resveratrol via the transport of oxidation metabolites into the vacuole is likely. The vacuole is often important in higher plants for detoxification of secondary metabolites. In plants, detoxification processes involve, at first, oxidation, reduction, or hydrolysis of the toxic product, followed by its conjugation to malonyl or glycosyl moieties by transferases and, finally, storage in the vacuole (33). Detoxification processes have also been described for fungi (44). The accumulation of resveratrol oxidation products in the vacuolar compartment of *B. cinerea* conidia could thus correspond to a detoxification process of this compound. Marbach et al. (32) strongly suggest that laccase is produced by *B. cinerea* conidia in order to deactivate plant defense mechanisms. The ability of *B. cinerea* conidia to metabolize resveratrol could thus contribute to creating a means of survival for conidia.

Since we observed the accumulation of resveratrol metabolites in the vacuole of *B. cinerea* conidia in relation to laccase activity, we wished to verify the localization of this enzyme in the fungal cell. Therefore, a cytochemical reaction using syringaldazine as a substrate for laccase (10,14) was done. Syringaldazine produces unique electron-opaque deposits after oxidation that are visible by transmission electron microscopy (15). We observed the same electron-opaque deposits when syringaldazine was used as the substrate for laccase (Fig. 3). Past experiments indicate that laccase was located predominantly in the cytoplasm (15) and also in the cell wall and surrounding mucilages (which is expected, because laccase is an extracellular enzyme) (36). Neither laccase nor syringaldazine deposits were detected at the vacuolar level. Our experiments show that laccase activity is present within the conidial cell and its cytoplasm (not in vacuoles) (Fig. 3), and this is in agreement with the findings of other investigators. Thus, the laccase-mediated oxidation of stilbenes takes place in the cytoplasm, and the resulting products later enter the vacuole where they accumulate, resulting in visible discoloration within the vacuole.

ACKNOWLEDGMENTS

We thank F. Marty, who enabled the realization of the transmission electron microscopy study at the Laboratoire de Phytobiologie Cellulaire, Université de Bourgogne, Dijon, France; D. Marty for her technical assistance; L. A. Weston at the University of Kentucky and L. M. Birden, ENSAM, Cluny, Burgundy, France, for reviewing the manuscript; and S. Debord and S. Kindt at the Laboratoire des Sciences de la Vigne, Université de Bourgogne, Dijon, France, and J. Relot at the Centre de Microscopie Appliquée á la Biologie, Dijon, France, for technical assistance.

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