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ORIGINAL RESEARCH ARTICLE

Fungitoxic role of endogenous eugenol in the hybrid grapevine cultivar Baco blanc resistant to *Botrytis cinerea*

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ABSTRACT

Eugenol (2-methoxy-4-(2-propenyl)-phenol), widely spread in various plants, notably clove, basil and bay, is a well-known antifungal and antibiotic molecule that is abundant in the hybrid grapevine cultivar Baco blanc (*Vitis vinifera* × *Vitis riparia* × *Vitis labrusca*). This variety, created by François Baco (19th century), is confirmed in this study as highly resistant to *Botrytis cinerea* by comparing fruit rot incidence and severity with two *Vitis vinifera* cultivars: Folle Blanche and Ugni Blanc. According to two major antibiosis modes of action, i.e., direct or volatile, this study demonstrated the efficiency of eugenol *in vitro* by also investigating precisely the effect on *B. cinerea* of small concentrations of eugenol, 3 to 4 ppm, corresponding to IC10. Moreover, the vapour-inhibiting effect was shown to be highly powerful. The total eugenol concentration peaked at the veraison stage, exceeding 1000 $\mu g \times kg^{-1}$ in the skin of Baco blanc berry under our conditions. At this point, leaf removal in the bunch zone induced a significant increase in eugenol (32 %), from 1118 to 1478 $\mu g \times kg^{-1}$, which was also associated with a significant decrease in *B. cinerea* infection in the vineyard. Thus, for the first time, eugenol, as an endogenous molecule of Baco blanc, was clearly demonstrated to be an inducible compound in the vineyard. Furthermore, significant intravarietal variability in eugenol concentrations according to the Baco blanc clone was demonstrated to be associated with significant differences in fruit susceptibility to the plant pathogen assessed in biotests. Interestingly, in keeping with fruit ontogenic resistance, a significant negative correlation was established between the technological maturity of berries and the total eugenol content in the berry skin. Finally, the time-progress study of the two biochemical forms of eugenol (bound *vs.* free eugenol) allowed us to hypothesise the effectiveness against the plant pathogen of some precursor forms of eugenol, and the corresponding biochemical structures are currently being investigated. Thus, eugenol appears to be a key biochemical marker of ontogenic resistance in the hybrid cultivar Baco blanc.

KEYWORDS: Armagnac, *Botrytis* bunch rot, grey mould, antibiosis, biocontrol, ontogenic resistance

INTRODUCTION

Baco blanc is a hybrid grapevine variety created by the French scholar François Baco at the end of the 19th century by crossing the two cultivars Folle Blanche (*Vitis vinifera*) and Noah (*Vitis riparia* × *Vitis labrusca*) (Baco, 1925, 1926). This hybrid grapevine was produced to resist phylloxera (*Daktulosphaira vitifoliae)*, first but also to be tolerant, at least partially, to other major plant diseases such as powdery and downy mildew and black rot, Baco blanc is now recognised as resistant to *Botrytis cinerea* (Galet, 2015). During the 20th century, it was kept and cultivated, although many bans in France targeted it as the other grapevine hybrid cultivars. Baco blanc is also known as a "distiller-destined variety" since it is essentially intended for the production of Armagnac wine spirit, where it is the basis for over 30 % of Armagnac production. Relatively few studies have focused on its distillation qualities but not on its pathological features accounting for its resistance traits (Bertsch, 1992; Hervé, 1996; Pedneault and Provost, 2016). Recently, and very interestingly, Franc *et al*. (2023) highlighted the high amount of eugenol in several tissues of Baco blanc and derived products, including grapevine shoots, leaves, grapes, wine and wine spirit unaged in barrels. This confirmed the possible presence of eugenol in hybrid vines that has already been noted (Mansfield & Vickers, 2009; Sun *et al*., 2011a; Sun *et al*., 2011b). Franc *et al*. (2023) also showed that a large part of eugenol could be released by enzymatic hydrolysis with a glycosidase, indicating the presence of eugenol as glycoconjugates in must, berry (skin and pulp), leaf and shoot of Baco blanc.

Eugenol is a well-known and well-studied phenylpropenic compound extracted mostly from the clove tree (*Eugenia caryophyllata*), whose cloves have been and still are the subject of lucrative trade (Kamatou *et al*., 2012). Its multiple biological properties include the following: antibacterial, antifungal, antiviral, antihelmintic, anti-inflammatory, analgesic, antioxidant, anticancer, and antimutagenic properties (Kamatou *et al*., 2012). Effective against several microorganisms, its action against *B. cinerea* has already been highlighted, particularly *in vitro*, where the efficacy of eugenol measured by the IC50 can significantly vary depending on the *in vitro* conditions used (Amiri *et al*., 2008; Combrinck *et al*., 2011; Fedele *et al*., 2020; Olea *et al*., 2019; Šernaitė *et al*., 2020; Velázquez-Nuñez *et al*., 2013; Wang *et al*., 2010).

Responsible for grey rot (Botrytis bunch rot BBR) and noble rot in vineyards, *B. cinerea* is a necrotrophic multihost fungus that is well-known to winegrowers as such (Amiri *et al*., 2008; Combrinck *et al*., 2011; Fedele *et al*., 2020; Olea *et al*., 2019; Šernaitė *et al*., 2020; Wang *et al*., 2010). At harvest, a BBR severity threshold as low as 5 % has been demonstrated to lead to irreversible organoleptic losses in the wine produced (Ky *et al*., 2012). The decrease in ontogenic resistance of grapevine fruit has been well characterised during berry development and maturation. However, further studies concerning the chemical composition of these organs may allow scientists to better understand the biochemical grounds of such a resistance pattern to the fungus (Deytieux-Belleau *et al*., 2009). Currently, the control of BBR is mainly carried out by using synthetic fungicides. However, public policies are increasingly tending to restrict the use of such synthetic pesticides, which are counterproductive in terms of fungal resistance and harmful to human health and/or the environment (Fenner *et al*., 2013). Other control means of prime importance are effective against *B. cinerea* based on i) prophylactic measures (Pañitrur-De la Fuente *et al*., 2020), ii) plant extracts, microbial suppression and/or chemical inducers of defence mechanisms (Elmer and Reglinski, 2006) and iii) biochemical pesticides—eugenol is incorporated as an active ingredient, together with geraniol and thymol in Mevalone® registered in France against *B. cinerea* (Fedele *et al*., 2020).

This study aims, first, to confirm the efficacy of eugenol against *B. cinerea* under *in vitro* conditions and to better assess its antifungal mode of action (direct antibiosis and/or volatile effect). Second, this study contextualises the antifungal property of eugenol in the case of Baco blanc by considering its varietal, clonal and ontogenic resistance to the fungus. In particular, it was necessary to show for the first time the eugenol concentration time-progress, accumulation and/or concentration variability in i) several clones of the cultivar and ii) in the berry skin according to fruit development and maturation. Finally, the inducibility of eugenol was investigated following leaf removal in the grape cluster zone and according to the known negative effect of this cultural practice on BBR infection.

MATERIALS AND METHODS

1. Experimental vineyards

The first site, "Domaine d'Ognoas" (43°54'07"N, 0°16'12"W), is situated in Arthez-d'Armagnac (Landes, Occitanie, France) in the "Bas-Armagnac" denomination. A Baco blanc collection plot, CLONES40 (43°53'34"N, 0°15'41"W), included in the Domaine d'Ognoas, was planted in 2013 on Gravesac rootstock according to a Latin square experimental design of 6 clones of Baco blanc, including the only one authorised in the French catalogue (clone 1086). In 2021 (the study year), no anti-*Botrytis* fungicide was applied to these vines.

The second site, "Château de Mons" (43°56'24"N, 0°26'45"E), is situated in Caussens (Gers, Occitanie, France) in the "Armagnac-Ténarèze" denomination. Two plots were studied as follows: i) a cultivar conservatory, named CONS32, and ii) a premultiplication plot, PREMS32, planted with the Baco blanc 1086 clone and different Ugni Blanc clones. As mentioned above, no anti-*Botrytis* treatment was applied to these vines in 2021.

2. Fungal Material

Different *B. cinerea* isolates were used, including i) strain "i" 213, i.e., a mono-spore strain from INRAE collection, isolated from grapevine leaf in 1998 (Martinez *et al.,* 2003),

ii) strain "j", i.e., strain 213 artificially inoculated and then reisolated from Baco blanc plant material, iii) isolate "P" originating at harvest 2020 from naturally infected berries in the PREMS32 plot, iv) isolate "V" Vitadapt 52c, i.e., populational isolate from naturally infected mature berries in a conservatory INRAE plot in La Grande Ferrade, Villenave d'Ornon, France and v) isolate "K", i.e., isolated at harvest 2020 from naturally infected berries in the CONS32 plot.

3. *In vitro* tests

3.1. Chemicals

Five eugenol (Sigma–Aldrich, Saint-Louis, Missouri, United States of America) solutions were prepared, from 1 to 10,000 ppm, in ethanol/water solution 0.3:1 (v/v). Two control solutions were prepared, sterile water and ethanol/water solution 0.3:1 (v/v).

3.2. Test preparation

Solid malt agar (15 g×L^{-1}, Crisomalt, Materne, France) medium was prepared and poured into 85 mm diam. Petri dishes. Two modes of action of eugenol were tested. For the direct mode of action, 500 µL of eugenol or control solution was spread on the whole available medium area with a single-use microbiological rake, and then a mycelial *B. cinerea* plug (4 mm in diam.) was deposited at the dish centre before sealing the dish with a polyvinyl chloride stretch film. For the mode of action by vapour, the plug was deposited at the dish centre, and the dish was then turned over. Then, 500 µL of eugenol or control solution was spread on the dish lid, and the inverted dish was placed over the lid and sealed as described above. All the dishes were incubated at 21 °C in the dark. In this experiment, *B. cinerea* isolates i and P of the plant pathogen were used.

3.3. Evaluation of *B. cinerea* development and calculation of inhibition concentration (IC)

After four days of incubation (4 dpi), two orthogonal diameters of every *B. cinerea* mycelial colony were measured. The mycelial area was calculated using an ellipse area equation as follows:

$$S = \pi \times (\text{diam. a}/2) \times (\text{diam. b}/2)$$

The calculation of ICs was carried out as previously published (Olea *et al*., 2019).

4. *In vivo* biotests on grapevine berries

Two biotests were performed with the following major characteristics (Table 1).

4.1. Bunch sampling and preparation

Only rank one or two bunches, i.e., bunches located at the base of the shoots, were collected in the different experimental plots at different phenological stages (according to the experiment: see Section 3. "Field trials"). The entire bunches were washed twice for 10 min in agitated tap water supplemented with Tween® 80 (Croda International plc, Snaith, UK), disinfected in a sodium hypochlorite solution (50 g×L^{-1}; pH adjusted at 7 with acetic acid), and finally rinsed twice with sterile water. Then, 500 to 1000 berries that were, uninjured, uniform in size and colour, and with the pedicel attached were selected. Fifteen berries were then placed on an aluminium grid in a closed plastic box (19 cm × 13 cm × 4 cm) that was used as an incubation humid chamber (100 % RH) and filled with 100 mL of sterile water at the box bottom. For every *B. cinerea* isolate, berries were inoculated individually by placing one 6-day-old mycelial plug at the berry equator with mycelium in contact with the fruit skin. Finally, the berries were incubated at 21 °C in the dark.

4.2. Evaluation of *B. cinerea* development

After incubation for seven days, incidence (% of affected berries) and severity (% of berry area affected) considering rotting and sporulation symptoms were visually assessed. Every berry was visually assessed as one replicate (= 15 replicates per box).

4.3. Quantification of *B. cinerea* spore number

After incubation for 14 days, only entire botrytised berries (severity of 100 % rot and/or sporulation) were placed in

TABLE 1. Characteristics of the biotests presented in this study.

Biotests characteristics	Biotest 1	Biotest 2
Studied cultivars	Baco blanc Folle Blanche Ugni Blanc	Baco blanc clones
Berries origin (plot concerned)	PREMS32 and CONS32	CLONES40
Number of pathogeny box	36	72
Number of berries	540	1080
Isolates used	i	I and P
Days of incubation	5	7
Incubation temperature (°C)	21	21
Relative Humidity (%)	100	100

an Erlenmeyer flask with 50 mL of sterile water and two drops of Tween® 80. After shaking at 350 rpm for five minutes on an orbital shaker (PSU-10i Orbital Shaking Platform, Grant Instruments, Cambridge, UK), the liquid phase was filtered through 100 µm nylon filters (Falcon®, VWR International, Radnor, Pennsylvania, United States of America), recovered into 40 mL bottles (Fisher Scientific, Waltham, Massachusetts, USA) and stored at –20 °C. Spore suspensions were then refiltered (second filtration) under a vacuum with a 20 µm filter (Millipore Corporation, Burlington, Massachusetts, USA). Then, spore suspensions were diluted by half with Isoton™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and spore quantification was performed with a Scepter™ 2.0 Cell Counter (Millipore Corporation, Burlington, Massachusetts, USA).

5. Field trials: experimental design and characteristics

In 2021, a one-year controlled leaf removal experiment in "Château de Mons" was carried out using one row of Baco blanc divided into three blocks. Every block consisted of seven consecutive control vines and eight consecutive leaf-removed vines. Four stages of grape development were determined using Growth Degree Days (GDD) accumulated from full flowering "50 % of flower caps fallen, in this study the Jun 2nd 2021 (Eichhorn & Lorenz, 1977; Hack *et al*., 1992; Lorenz *et al*., 1995): cluster closure (July 7th 2021, 377 GDD, BBCH code 77), véraison (Aug 10th 2021, 719.3 GDD, BBCH code 81), harvest (Sept 23rd 2021, 1199.9 GDD, BBCH code 89) and postharvest (Sept 29th 2021, 1253.3 GDD, BBCH code 91).

In 2021, a one-year experiment was conducted for monitoring Baco blanc clones in the study collection plot ("Domaine d'Ognoas") using the Latin square experimental design with six Baco blanc clones with ten consecutive vines per clone.

5.1. Field measurements

At the harvest stage, all the field measurements were performed on both plots, CLONES40 and PREMS32, as follows.

5.1.1. Normalised difference vegetation index (NDVI) for quantitatively assessing leaf removal

In viticulture, for vertical shoot positioned vines, the NDVI quantitatively represents the leaf area index (LAI) in the centre of the row, also taking into account the gap fraction of the canopy (Drissi *et al*., 2009; Pañitrur-De la Fuente *et al*., 2020). The NDVI was measured with a hand-held GreenSeeker® (Trimble, Sunnyvale, CA, USA) exactly as described by Drissi *et al*. (2009) and Pañitrur-De la Fuente *et al* (2020).

5.1.2. Nondestructive measurements on bunches

5.1.2.1. Crop load per grapevine

For each treatment, all bunches were counted according to the 153 Organisation Internationale de la Vigne et du Vin (OIV) protocol (OIV., 2014). The crop load of each treatment was divided by the grapevine number to calculate the crop load per grapevine.

5.1.2.2. Bunch compactness

Bunch compactness was evaluated visually by sorting all bunches in every treatment according to the compactness classes of the 204 OIV protocol (OIV., 2014). The OIV protocol was improved by using photographs, and the values attributed to each class were simplified (1-2-3-4-5 instead of 1-3-5-7-9).

5.1.2.3. Bunch contact rate (τ)

All isolated, lonely, bunches, i.e., one bunch not touching another bunch were counted in each treatment. Calculation of the bunch contact rate was performed as follows:

$$\tau = 1 - \text{(number of isolated bunches/total crop load)}$$

5.1.2.4. *Botrytis* infection visual evaluation

Botrytis infection was evaluated taking into account both BBR incidence and severity as previously described (Pañitrur-De la Fuente *et al*., 2020).

6. Quantification of biochemical compounds

6.1. Eugenol quantification in grape skins

6.1.1. Step 1: Preparation of grape skin powder

Approximately 50 berries—from each studied plot previously mentioned and at different phenological stages previously determined—were peeled and then dried on absorbent paper. Skins were kept at –25 °C before crushing with liquid nitrogen using a Retsch MM400 ball mill (Retsch France, Verder S.A.R.L., Eragny sur Oise, France). Fine skin powder was kept in liquid nitrogen until weighing 200 mg in a 15 mL Falcon® tube.

6.1.2. Step 2: Water extraction and enzymatic hydrolysis

Two kinds of samples were prepared: one to analyse the eugenol-free fraction by simple extraction with water and one to analyse the eugenol-bound fraction by enzymatic hydrolysis (Gunata *et al*., 1993). Skin powder weighed in a 15 mL Falcon® tube was added to 10 mL of Milli-Q water (water extraction) and placed on an IKA HS 501 digital reciprocating shaker (VWR International S.A. S, Fontenay-sous-Bois, France) for 24 h at 250 rpm. Alternatively, the 15 mL Falcon® tube containing skin powder was added to 10 mL of a citrate-phosphate buffer solution (0.1 M, pH 5) and 300 µL of a glycosidase-rich enzyme preparation: AR2000 (Gunata *et al*., 1993). The mixture was then placed in an SW22 water bath (Julabo GmbH, Seelbach, Germany) to incubate for 24 hours at 40 °C and 150 rpm.

Next, the water extract and the enzymatically hydrolysed extract were centrifuged with a High-Speed Refrigerated Centrifuge Himac CR22N (Hitachi Life-Sci, Tokyo, Japan) equipped with a fixed angle rotor R15A at 5000 rpm for five minutes to separate skin residues from water or buffer extract.

6.1.3. Step 3: HS-SPME-GC-MS analysis (Franc *et al.*, 2023)

Ten millilitres of the supernatant obtained after centrifugation was introduced into a 20 mL glass vial containing 3.5 g

of sodium chloride, and 20 µL of a solution of deuterated eugenol (50 mg×L^{-1} in 100 % ethanol) was added as the internal standard (IS). All samples were prepared in triplicate. GC–MS analysis was performed with the same method and instrument as described in Franc *et al*. (2023): an Agilent Technologies (Les Ulis, France) 7890 gas chromatograph system coupled to an Agilent Technologies (Les Ulis, France) 5975C quadrupole mass spectrometer equipped with a Gerstel (Müllheim/Ruhr, Germany) MPS autosampler. A BP-21 capillary column (50 m × 0.32 mm, 0.25 µm film thickness, SGE, Courtaboeuf, France) was used with helium N55 as the carrier gas (Messer France S.A. S, Suresnes, France).

6.2. Quantification of condensed tannins in grape berry skin

6.2.1. Step 1: Preparation of grape skin powder

Since tannins contained in grape skin are photo- and thermosensitive, iced grape berries were peeled to obtain at least 1.5 g of fresh skins in a bowl refreshed by liquid nitrogen to protect skins from oxidation. The skins were stored at –20 °C until crushing with a cryogrinder Freezer Mill 6875D (SPEX SamplePrep, Metuchen, New Jersey, USA).

6.2.2. Step 2: Extraction and quantification of condensed tannins from grape skin powder

The extraction protocol followed was that of Gagné *et al*. (2006) with slight modifications. The quantification principle is based on the Bate-Smith reaction: in an acidic matrix, the tannins (flavanol chains or procyanidins) are partially transformed into cyanidins by heating, which are then quantitatively assessed by spectrophotometry (Ribéreau-Gayon and Stonestreet, 1965). The present protocol is an adaptation by Gagné (2007).

6.3. Maturity analysis

6.3.1. Total Acidity (TA)

TA is used during grape maturation monitoring and stands for titratable acidity. Neutralisation of matrix acid functions with an alkaline solution was appreciated using an indicator dye, bromothymol blue (BTB) ($C_{27}H_{28}Br_2O_5S$), the colour of which is yellow at acidic pH, reaching pigeon-blue at pH 7 (OIV., 2014).

6.3.2. Sugar concentration

To analyse the sugar concentration of the must, we used a digital refractometer Milwaukee MA871 (Milwaukee Instruments, Rocky Mount, North Carolina, USA) based on the Snell-Descartes law. The results were expressed in Brix degree (°B) converted into the sugar concentration expressed in g×L^{-1}.

Sugar concentrations can be expressed per berry. Expressing the concentration in this way makes the volume of the berries irrelevant, eliminating variability caused by irrigation or heavy rainfall (Deloire, 2011).

6.3.3. Maturity index

Technological maturity, with its standard index (TMI = Technology Maturity Index), was defined by the sugar:acid ratio.

7. Statistical analyses

Statistical analyses (Shapiro–Wilk normality test, Levene homoscedasticity test, two-way ANOVA, Kruskal–Wallis test (KW), Wilcoxon–Mann–Whitney test (WMW), Newman–Keuls *post hoc* test (NK), Fisher's Least Significant Difference (LSD) *post hoc* test) and plots were performed with rStudio software (version 2021.09.2). Specific use of adapted tests is indicated in the following figure captions.

RESULTS

1. Eugenol modes of action and efficacy in vitro against *B. cinerea*

The *in vitro* experiment confirmed the efficiency of eugenol against the two different isolates of *B cinerea.*

First, in terms of direct contact antibiosis and possibly also some volatile effect, with the isolate "P", the three mycelial inhibition concentrations studied, i.e., The IC10, IC50 and IC100 reached 0.48, 2.42, and 4.89 $\log_{10}$ ppm, respectively (Figure 1A). For strain "j", the corresponding values were 0.47, 2.38 and 4.79 $\log_{10}$ ppm, respectively. There was no significant isolate effect (ANOVA, p-value = 0.96. However, the differences in eugenol concentrations (expressed in $\log_{10}$(ppm)) corresponding to the different inhibition rates were highly significant (ANOVA, p-value = 8.6 10^{-14}). An increased eugenol concentration significantly inhibited *B. cinerea* mycelial growth. Moreover, there was no significant interaction between the two main effects, i.e., iCs and the *B. cinerea* isolate (ANOVA, p-value = 0.99). A linear regression was applied using the IC average of the 2 isolates, with Equation y = 0.048x and R^2 = 0.99.

Second, for the volatile action only of eugenol, the iCs were as follows for the *B. cinerea* isolate "P": 0.5, 2.5 and 5.06 $\log_{10}$ ppm, respectively (Figure 1B). The corresponding linear regression was with the Equation y = 0.05x (R^2 = 0.99). The iCs for strain "j" were 0.67, 3.46 and 7.11 $\log_{10}$ ppm, respectively, leading to the linear regression Equation y = 0.071x (R^2 = 0.99). There were significant differences in $\log_{10}$(ppm) between the eugenol iCs (ANOVA, p-value = 1.01 × 10^{-12}). Moreover, there was a significant difference between the two plant pathogen isolates (ANOVA, p-value = 1.15 × 10^{-4}). Finally, there was no significant interaction between the two main effects: iCs and *B. cinerea* isolates (ANOVA, p-value = 0.24).

2. Eugenol content in berry skin related to *B. cinerea* susceptibility in different cultivars and/or Baco blanc clones

2.1. Partial resistance of Baco blanc to *B. cinerea* and differences among cultivars

The first assay, Biotest 1, using mature berries confirmed the higher resistance of Baco blanc compared to the two

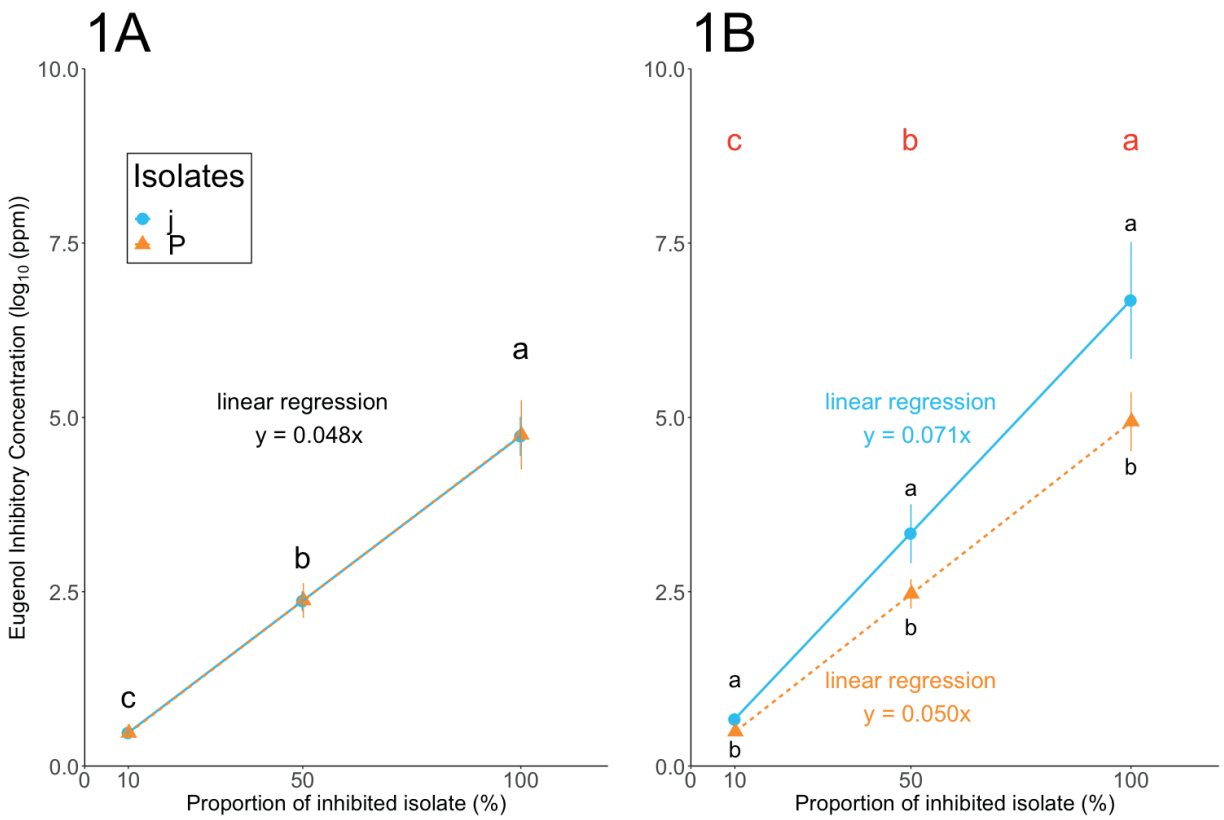


FIGURE 1. Concentrations of eugenol (expressed in log10 of ppm) corresponding to 10 %, 50 % and 100 % of mycelial inhibition of two plant pathogen isolates.

Each dot represents the average of 3 replicates values with standard deviation bar. Mean values with different letters are significantly different (p value < 0.05). A, concentrations of eugenol corresponding to 10 %, 50 % and 100% of mycelial inhibition of two plant pathogen isolates by direct application of eugenol. Statistical groups obtained with two-ways ANOVA (p value = 8.6 10-14, F value = 896.0, df = 2) and Newman-Keuls post hoc test. B, concentrations of eugenol corresponding to 10 %, 50 % and 100 % of mycelial inhibition of two plant pathogen isolates by volatile action of eugenol. Statistical groups of ICs obtained with two-ways ANOVA (p value = 1.0×10-12, F value = 593.0, df = 2) and Newman-Keuls post hoc test. Mean values with different red letters are significantly different (p value < 0.05) Statistical groups of isolates obtained with two-ways ANOVA (p value = 1.15×10-4, F value = 31.45, df = 1) and Newman-Keuls post hoc test. Mean values with different black letters are significantly different (p value < 0.05).

other key Armagnac varieties tested (*V. vinifera*), i.e., Folle Blanche and Ugni Blanc (Figure 2 A,B).

Considering rotting severity in the berry area (Figure 2 A), Folle blanche was significantly the most susceptible cultivar (mean rot area of 87.7 %), whereas Baco blanc from the CONS32 plot was significantly the most resistant cultivar (21.6 % severity). The other Baco blanc berries, originating from the PREMS32 plot, were intermediate in susceptibility, i.e., rotting severity of 38.3 %. They were significantly different from the Baco blanc berries in CONS32 but not significantly different from the Ugni blanc fruit, i.e., 42.3 % (KW p-value $\chi^2 = 5.42 \times 10^{-5}$)

Similarly, considering sporulation severity (Figure 2B), Folle blanche was, significantly, the most susceptible and sporulating cultivar (48.1 %). Baco blanc from CONS32 was significantly the most resistant cultivar (9.0 %). The Baco blanc in PREMS32 (16.5 %) was intermediate, significantly different from the Baco blanc in CONS32 and not significantly different from the Ugni blanc, i.e., 15.9 % (KW, p-value $\chi^2 = 1.14 \times 10^{-3}$).

2.2. Time-progress of eugenol concentrations in free and bound forms during grape berry development and effect of leaf removal

The 2021 one-year controlled leaf removal experiment allowed us to i) quantify total, free and bound eugenol contents at four key stages of berry development and ii) show the significant effect of leaf removal (Figure 3A,B).

The induction of total eugenol following leaf removal in the bunch zone is shown in Figure 3A. At the véraison stage, the total eugenol content in the berry skin from leaf-removed vines, i.e., 1478 µg×kg^{-1}, was significantly higher than that in the control vines, reaching 1118 µg×kg^{-1} (WMW, p-value = 4.96×10^{-4}, W = 129). Similarly, at harvest, the total eugenol content in the berry skin from leaf-removed vines, 1078 µg×kg^{-1}, was significantly higher than in the control vines, i.e., 778 µg×kg^{-1} (WMW, p-value = 3.33×10^{-5}, W = 137). However, at bunch closure, there was no significant difference between the total eugenol content in the berry skin from leaf-removed and control vines, i.e., 165 µg×kg^{-1} (WMW, p-value = 1, W = 72), and at the postharvest stage,

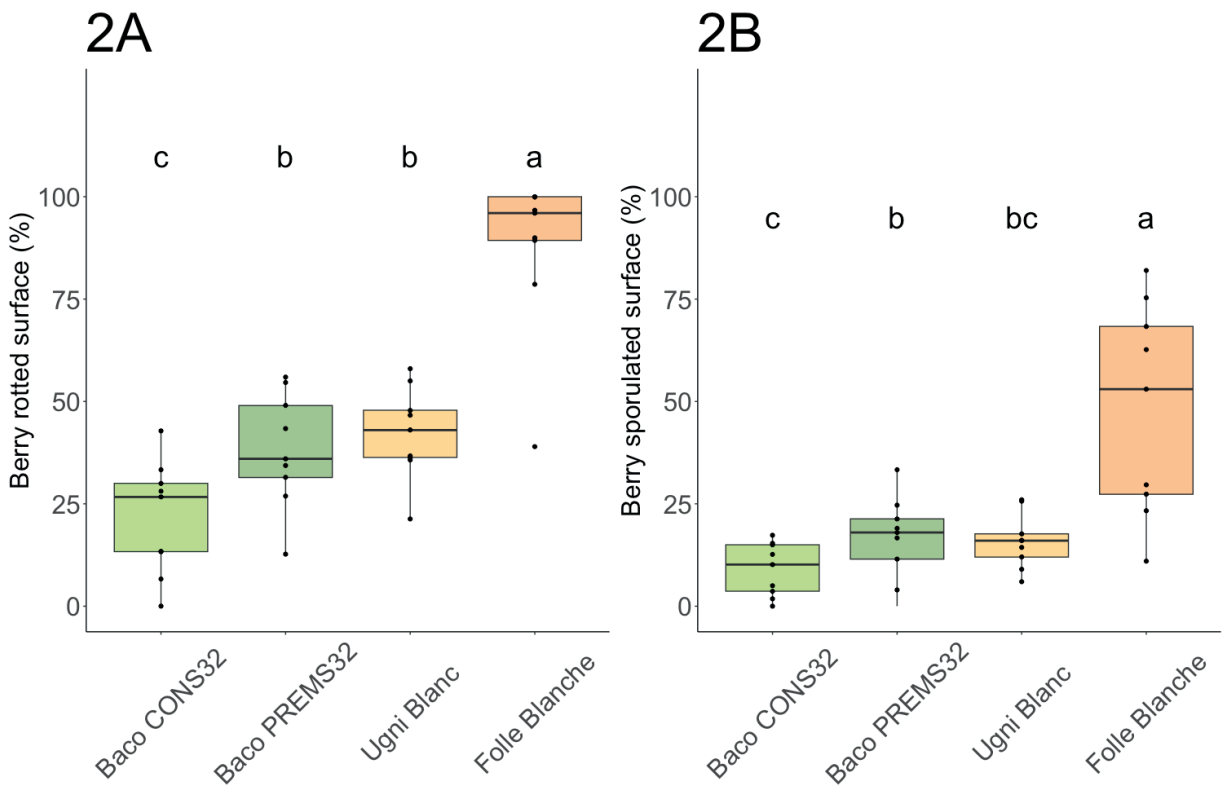


FIGURE 2. *Botrytis cinerea* symptoms severities of four grape varieties in biotest conditions. A, rotting severity of four grape varieties and B, sporulation severity of the same four grape varieties.

Each dot in boxplots represents a replicate. Modality Baco CONS32 represents Baco blanc grapes sampled in CONS32 plot; modality Baco PREMS32 represents Baco blanc grapes sampled in PREMS32 plot. Grape varieties with different letters are significantly different (p value <0.05). For A, statistical groups obtained with Kruskal-Wallis test (p value $\chi2$ = 5.42×10-5)and Fisher's Least Significant Difference post hoc test (t-Student = 2.04, minimal significant difference = 6.35).And for B, statistical groups obtained with Kruskal-Wallis test (p value $\chi2$ = 1.14×10-3) and Fisher's Least Significant Difference post hoc test (t-Student = 2.04, minimal significant difference = 7.80).

there was no significant difference either in the total eugenol content, i.e., 223 or 245 µg×kg^{-1}, (WMW, p-value = 0.14, W = 98). Between the first two stages, the total eugenol concentration in the berry skin increased by 797 % in leaf-removed vines and by 579 % in the control vines. Finally, at the end of berry development, i.e., between harvest and the postharvest stage, the total eugenol concentration in berry skin decreased by 77 % in leaf-removed vines and by 71 % in the control vines.

The time progress of bound and free eugenol following leaf removal in the bunch zone is shown in Figure 3B. At the véraison stage, the bound eugenol content in the berry skin from leaf-removed vines, i.e., 1375 µg×kg^{-1}, was significantly higher than that in the control vines: 1020 µg×kg^{-1} (WMW, p-value = 2.3 × 10^{-3}, W = 123). Similarly, at harvest, the bound eugenol content in the berry skin from leaf-removed vines, 108.68 µg×kg^{-1}, was higher than in the control vines, i.e., 62 µg×kg^{-1} (WMW, p-value = 1.7 × 10^{-2}, W = 113). At harvest too, the free eugenol content in the berry skin from leaf-removed vines, 969 µg×kg^{-1}, was higher than in the control vines, i.e., 716 µg×kg^{-1} (WMW, p-value = 2.7 × 10^{-4}, W = 131). At the postharvest stage, the bound eugenol content in the berry skin from leaf-removed vines, 21 µg×kg^{-1}, was higher than that in the control vines, i.e., 7 µg×kg^{-1} (WMW, p-value = 8.5 × 10^{-3}, W = 118). However, at bunch closure, there was no significant difference between the bound eugenol content in berry skin from leaf-removed and control vines, i.e., 155 µg×kg^{-1} (WMW, p-value = 1, W = 72). There was no significant difference either in the free eugenol content, i.e., 10 µg×kg^{-1} (WMW, p-value = 1, W = 72). At the véraison stage, there was no significant difference between the free eugenol content in berry skin from leaf-removed vines and control vines, i.e., 104 or 99 µg×kg^{-1} (WMW, p-value = 0.977, W = 71). At the postharvest stage, there was no significant difference between the free eugenol content following leaf removal, i.e., 224 or 216 µg×kg^{-1} (WMW, p-value = 0.59, W = 82). Between the first two stages, the bound eugenol content in the berry skin increased by 769 % for leaf-removed vines and by 576 % for control vines. The free eugenol content in the berry skin increased by 967 % for leaf-removed vines and by 916 % for control vines between the véraison and harvest stages. Finally, at the end of berry development, between the harvest and postharvest stages, the bound eugenol concentration in berry skin decreased by 81 % for leaf-removed vines and by 88 % for control vines. The free eugenol content in berry skin

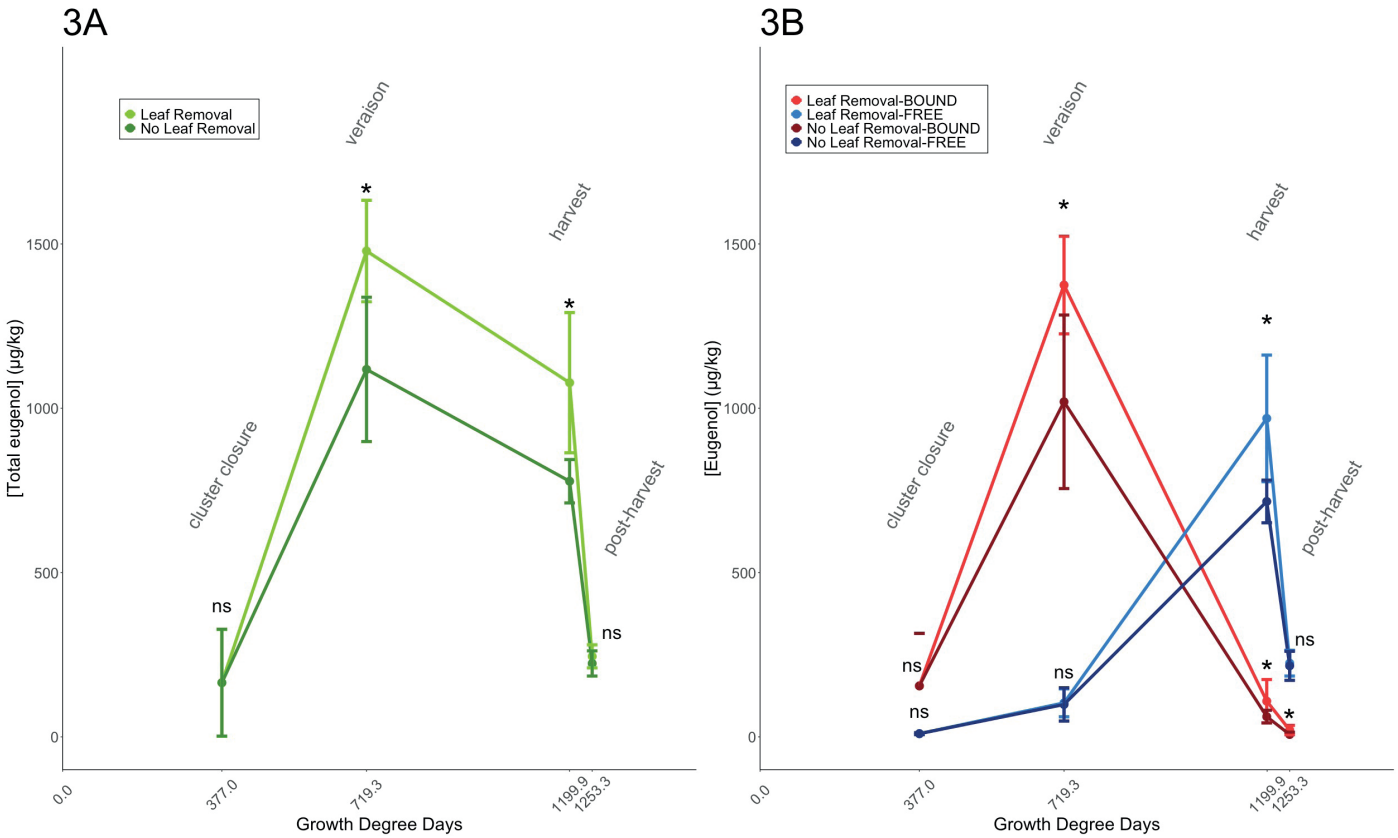


FIGURE 3. Time-progress of eugenol concentrations in free and bound forms during grape berry development and effect of leaf removal. A, time-progress of total eugenol content during grape berry development and effect of leaf removal.

Stars represent significant differences between leaf-removed vines and no leaf-removed vines obtained by Wilcoxon-Mann-Whitney test. B, time-progress of free and bound eugenol contents during grape berry development and effect of leaf removal. Stars represent significant differences between leaf-removed vines and no leaf-removed vines for free eugenol on one hand and between leaf-removed vines and no leaf-removed vines for bound eugenol obtained by Wilcoxon-Mann-Whitney test. For each value of Growth Degree Days, a phenological stage is associated.

decreased by 77 % for leaf-removed vines and by 70 % for control vines.

2.3. Clonal variability within Baco blanc: eugenol content and susceptibility to the plant pathogen

Bound and free eugenol concentrations were evaluated in the berry skin of six Baco blanc clones at the harvest stage, including c1086 authorised in the French catalogue of grape varieties.

All clones presented a higher free eugenol concentration than the bound eugenol concentration in berry skin, except for the E12 clone (Figure 4). Considering free eugenol (Figure 4), the E22 clone showed the highest concentration of 2580 µg×kg^{-1}, while the E12 clone exhibited a minimal concentration of 1039 µg×kg^{-1}. Statistically intermediate clones were E36 (1939 µg×kg^{-1}), E30 (1853 µg×kg^{-1}), E15 (1782 µg×kg^{-1}) and c1086 (1445 µg×kg^{-1}) (ANOVA, p value = 6.9 × 10^{-2}). There was no significant difference between the six clones in bound eugenol (Figure 4). However, berry skin of the E36 clone tended to be the most concentrated at 1600 µg×kg^{-1}, followed by E30 at 1549 µg×kg^{-1}, E12 at 1275 µg×kg^{-1}, E15 at 1225 µg×kg^{-1}, and E22 at 1173 µg×kg-1, and c1086 was the least concentrated at 1081 µg×kg^{-1} (ANOVA, p-value = 0.95).

2.4. Differential susceptibility of the six Baco blanc clones to the plant pathogen

In the second *in vivo* biotest, biotest 2, using the populational isolate "P" (Figure 5 A), the proportion of rotted berries (incidence) was not significantly different between clones (ANOVA, p-value = 0.15), with incidence values ranging from 81.0 % (E22) to 42.1 % (E12). For rot severity, i.e., mean rotted area (%) at the berry surface (Figure 5 A), the following significant order was observed with a similar incidence hierarchy: the E22 clone was the most susceptible (57.5 %), while the E12 clone was the least susceptible (20.4 %). Clones E36, c1086, E15 and E30 were intermediate with respective severities of 46.9 %, 31.2 %, 28.1 % and 27.9 %. (ANOVA, p-value = 3.6 × 10^{-2}) while the E12 clone was the least susceptible (20.4 %).

Concerning the collection strain « j » (Figure 5 B), the incidence was significantly different between clones (KW, p-value χ^2 = 3.8 × 10^{-1}). The E36 clone was significantly the most susceptible (97.8 %), while the E12 clone was

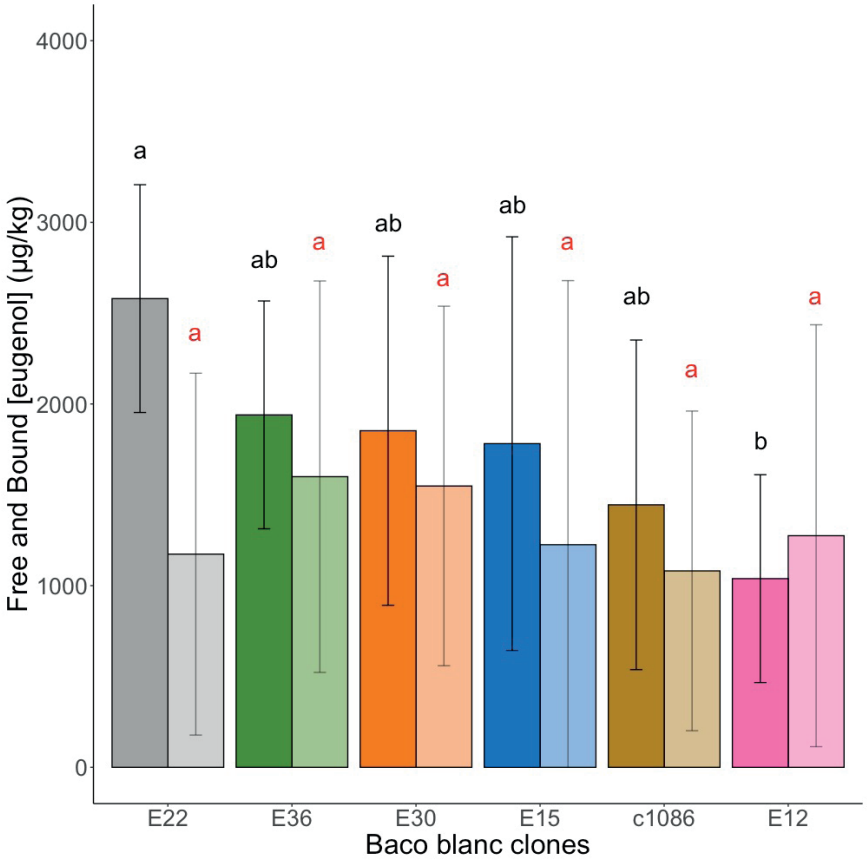


FIGURE 4. Means free and bound eugenol content in six Baco blanc clones including the only one authorized in PDO Armagnac.

Solid color bar plots represent free eugenol content and clear color bar plots represent bound eugenol content. Error bar on each bar plot is ± standard deviation. Mean free eugenol contents in clones with different black letters are significantly different. Statistical groups are obtained by ANOVA (p value = 6.9 10-2, F value = 2.31, df = 5) followed by Newman-Keuls' test. Mean bound eugenol contents in clones with different red letters are significantly different. Statistical groups are obtained by ANOVA (ANOVA, p value = 0.95, F value = 0.215, df = 5).Quantification of eugenol forms performed on samples from CLONES40 plot at harvest (8th September, 1084.7 GDD).

again significantly the least susceptible clone (78.3 %). Clones E22, E15, c1086 and E30 were intermediary clones with respective incidences of 94.4, 93.3, 91.1 and 90.0 %. The severity of rot (Figure 5 B) was not significantly different between clones (ANOVA, p-value = 0.20), with incidence values ranging from 76.6 % (E22) to 51.9 % (E12).

The sporulation severity using the populational isolate "P" (Figure 5 C), i.e., mean sporulated area (%) at the berry surface was significantly different between clones (KW: p-value χ^2 = 0.2). The two clones E15 and c1086 were significantly the most susceptible ones, with respective severities of 21.0 and 20.5 %, while E22 was significantly the most resistant clone (6.9 %). Clones E30, E12 and E36 were intermediate in severity, with 21.7, 18.8 and 14.6 %, respectively. Considering the count of spores per berry, c1086 was significantly the most sporulating clone (178,621 spores per berry), while E15, E30, E36 and E22 were significantly the least sporulating clones, with 95,480, 95,480, 95,480 and 94,759 spores per berry, respectively. E12 was an intermediary clone with 117,472 spores per berry (KW: p-value $\chi^2 = 6.2 \times 10^{-2}$).

The sporulation severity with the collection strain "j" (Figure 5 D) was significantly different between clones (KW: p-value $\chi^2 = 2.0 \times 10^{-1}$). The two clones E12 and E30 were significantly the most susceptible (21.6 and 14.4 %, respectively), while E36 was the most resistant clone (5.9 %). Clones c1086, E15 and E22 were intermediate: 11.1, 9.4 and 8.7 %, respectively. Finally, concerning the count of spores per berry, there was a significant difference between clones (KW: p-value $+\chi^2 = 4.7 \times 10^{-3}$). Clone E22 was the least sporulating clone, with 44,713 spores per berry. Clones E30, E15, E36, c1086 and E12 presented 95,480, 95,480, 95,480, 71,385 and 65,934 spores per berry, respectively.

3. Maturity analysis of 6 Baco blanc clones

The harvest values of the maturity analyses in the six Baco blanc clones (Table 2) are mostly expressed in quantity per berry to avoid taking into account the effect of potential precipitation during the experiment. Regardless of the maturity variable considered, no significant difference was detected between the clones.

4. Relation between eugenol content and technological maturity in berry skin

We established significant negative correlations (p = 0.05) between the total eugenol content in berry skin and the

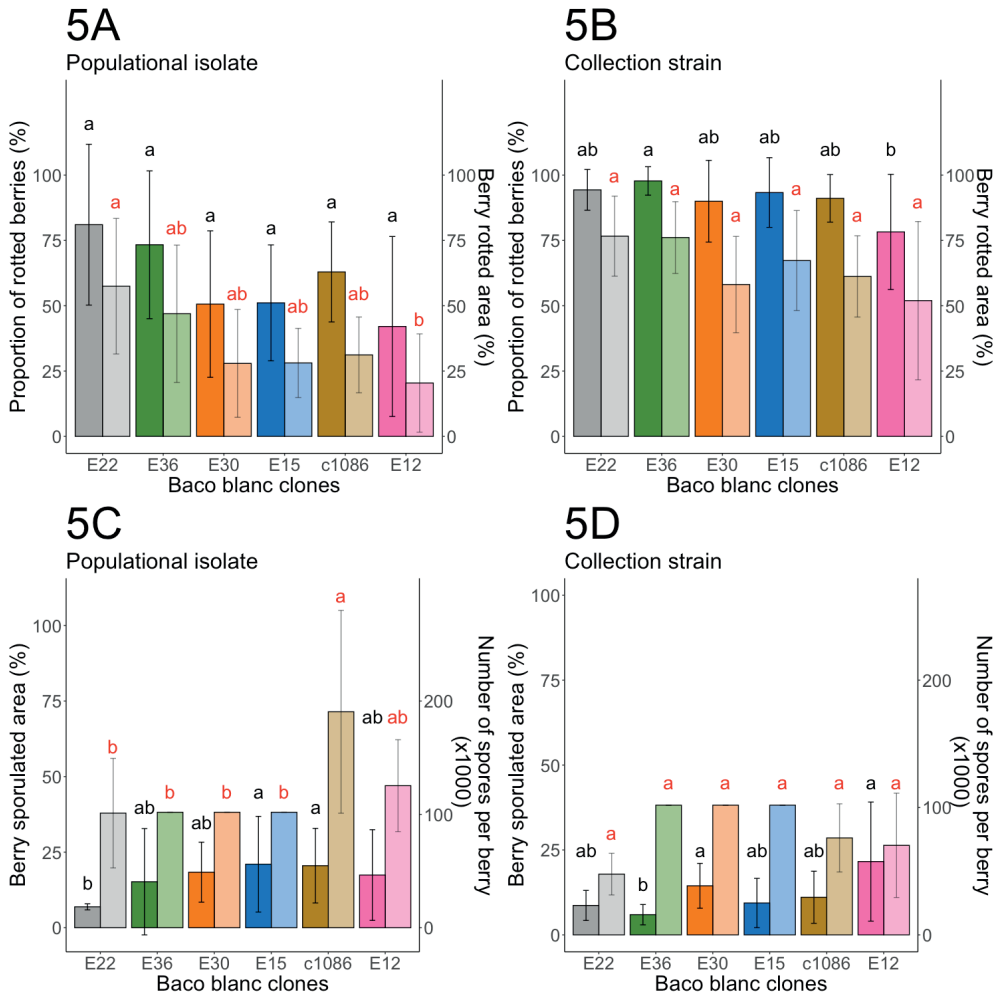


FIGURE 5. Evaluation of *Botrytis cinerea* symptoms on clones according to the isolate used during biotest 2.

On each graphic, error bars are ± standard deviation. For A (populational isolate) and B (collection strain), solid color bars represent mean of rotting incidence (%) on clones and transparent color bars mean of rotting severity (%) on clones. For A, rotting incidence means with different black letters are significantly different (ANOVA, p value = 0.15, F value = 1.8, df = 5), and rotting severity means with different red letter are significantly different (ANOVA, p value = 3.59 10-2, F value = 2.8, df = 5; followed by Newman-Keuls test). For B, rotting incidence means with different black letters are significantly different (KW, p value $\chi 2$ = 3.8×10-1, critical-value = 5.3, ddl = 5; LSD: t-Student = 2.0, minimal significant difference = 11.4) and rotting severity means with different red letters are significantly different (ANOVA, p value = 0.2, F value = 1.6, df = 5). For C (population isolate) and D (collection strain), solid color bars represent mean of sporulation severity (%) on clones and transparent color bars represent mean of spores per berry (×1000) on clones. For C, sporulation severity means with different black letters are significantly different (KW: p value $\chi 2$ = 0.2, critical-value = 7.7, df = 5; LSD: t-Student = 2.4, minimal significant difference = 11.8) and means of spores per berry (×1000) with different red letters are significantly different (KW: p value $\chi 2$ = 6.2×10-2, critical-value = 10.53, df = 5); LSD: t-Student = 2.0, minimal significant difference = 10.1). For D, sporulation severity means with different black letters are significantly different (KW: p value $\chi 2$ = 2,0×10-1, critical-value = 7.3, df = 5); LSD: t-Student = 2.0, minimal significant difference = 11.9) and means of spores per berry (×1000) with different red letters are significantly different (KW: p value $\chi 2$ = 4.7×10-3, critical-value = 16.9, df = 5); LSD: t-Student = 2.0, minimal significant difference = 8.9).

Technological Maturity Index TMI in the fruit (Figure 6). Overall, most interestingly, when all the data were pooled, the negative correlation was highly significant between the total eugenol content in berry skin and the TMI, i.e., R = –0.90 (R^2 = 0.81), p-value < 2.2 × 10^{-16}. (Figure 6). Thus, the riper the Baco blanc berries are, the lower the concentration of total eugenol quantified in their skin is.

By using the CLONES40 data only, the negative Pearson's correlation coefficient was very close to being significant, i.e., R = –0.89 (R^2 = 0.79), p-value = 0.05. For the PREMS32 data, Pearson's negative correlation coefficient was highly significant between the total eugenol content in berry skin and the TMI, i.e., R = –0.45 (R^2 = 0.20), p-value = 0.009.

5. Effect of leaf removal on *in naturae Botrytis* infection

For the BBR severity in Baco blanc in the vineyard (Figure 7), the leaf-removed vines showed a mean BBR severity of 6.3 %, which was significantly lower than the BBR severity in the control vines (no leaf-removal), reaching 19.3 % (KW: p-value $\chi 2$ = 0). Similarly, for the incidence of *B. cinerea* infection (Figure 7), leaf-removed vines presented a significantly reduced incidence, i.e., 69.8 %, *vs*. 89.3 %

TABLE 2. Maturity analyses performed on the six Baco blanc clones at the harvest stage (8th September, 1084.7 GDD).

Clones	Volume per berry (mL)	[Sugar] per berry (mg)	TA per berry (mg H_2SO_4)	Technological Maturity Index	[Tannins] (mg/g of berry skin)
E22	1.89^a ± 0.17	189^a ± 38	26^a ± 4	7^a ± 2	18.90^a ± 5.05
E15	1.69^a ± 0.23	177^a ± 34	22^a ± 4	8^a± 2	18.82^a ± 0.00
c1086	1.80^a ± 0.21	188^a ± 33	23^a ± 4	8^a ± 2	19.09^a ± 3.80
E30	1.89^a ± 0.20	199^a ± 22	25^a ± 4	8^a ± 1	18.82^a ± 0.00
E12	1.88^a ± 0.40	178^a ± 49	24^a ± 6	7^a ± 1	18.48^a ± 4.39
E36	1.84^a ± 0.27	195^a ± 34	24^a ± 5	8^a ± 2	18.82^a ± 0.00

Values are expressed by the mean of each indicator ± the standard deviation. Means with different letters are significantly different (ANOVA followed by SNK except for [tannins]: Kruskal-Wallis test).

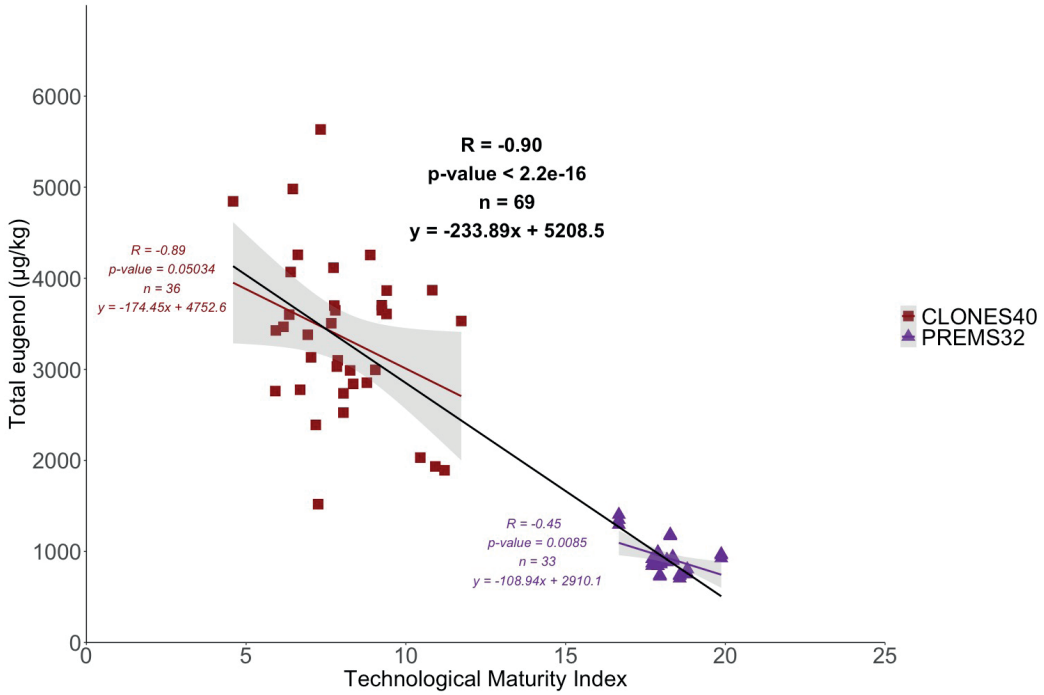


FIGURE 6. Correlation between Technological Maturity Index (TMI) and total eugenol content in grape berry skin.

Red dots and linear regression red line depict samples from CLONES40 plot. Purple dots and linear regression purple line depict samples from PREMS32 plot. The linear regression black line represents a general correlation (samples from the both plots). For each regression, the Pearson's R, the p value, the number of samples considered (n) and the equation of the linear regression (y = ax + b) are indicated next to the regression line. Eugenol quantification and TMI calculation were performed at the same stage, i.e., at harvest corresponding to Sept 23rd 2021, 1199.9 GDD and Sept 8th 2021, 1084.7 GDD, respectively.

in the control vines without any leaf removal (KW: p-value $\chi 2 = 5.14 \times 10^{-11}$).

6. Field measurements

Different field measurements, as described in the Materials and Methods section, were performed. These results are presented in the supplementary data (Supplementary Table 1). In most cases, no significant differences were identified between treatments except for two circumstances. First, in the PREMS32 plot, leaf-removed vines showed a significantly lower NDVI value than control vines. Second, in the CLONES40 plot, two clones presented a bunch compactness significantly different from the other clones.

DISCUSSION

1. Eugenol with different modes of action against *B. cinerea*

First, liquid eugenol, when brought in direct contact with the plant pathogen, showed a significant and marked antibiosis effect *in vitro*. It is very important to note that, under our experimental conditions, such a direct effect of eugenol also includes a vapour effect. The eugenol solution deposited on the agar evaporated partly in the Petri dish gaseous space. Therefore, liquid eugenol, under our conditions, included both modes of action, direct contact

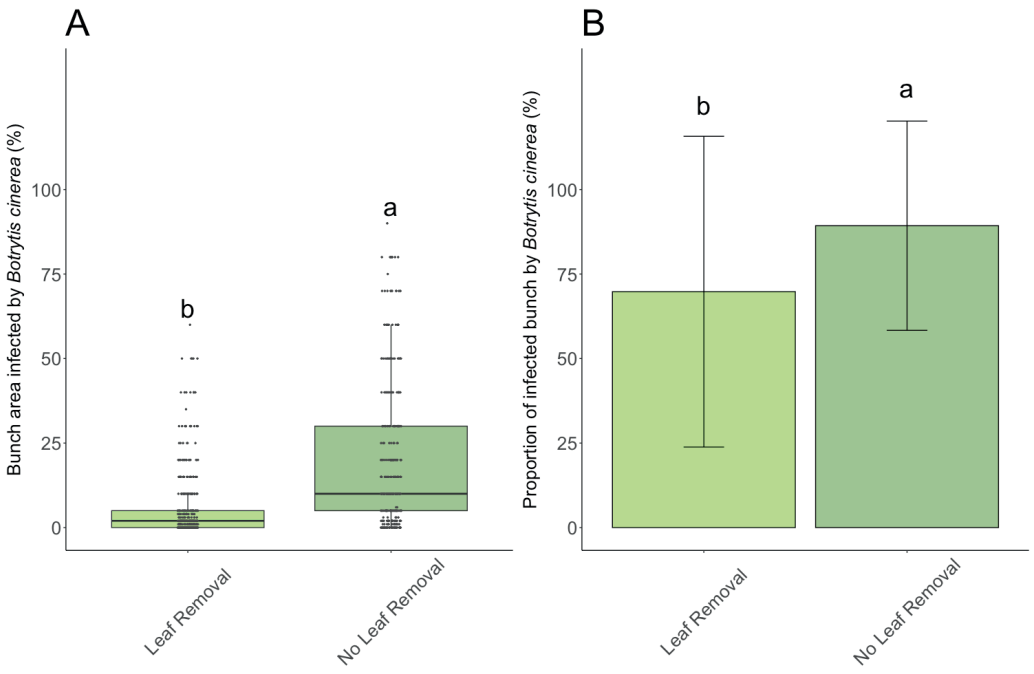


FIGURE 7. Evaluation of *Botrytis cinerea* symptoms in the vineyard as affected by leaf removal on the cv Baco blanc (harvest stage).

A, severity of infection (% of bunch surface infected) in grape bunches from leaf-removed vines and control vines. For each treatment, dots represent the number of considered bunches. Treatments with different letters are significantly different (KW: p value $\chi 2$ = 0, critical-value = 129.1, df = 1; LSD: t-Student = 1.9). B, incidence of infection (% of infected bunches) on grape bunches from leaf-removed vines and no leaf-removed vines. For each modality, bar represents mean of incidence and error bars are ± standard deviations. Means with different letters are significantly different (KW: p value $\chi 2$ = 5.1×10-11, critical-value = 43.1, df = 1; LSD t-Student = 1.9).

action *sensu stricto* and vapour action. This resulted in very close and reproducible IC50 values for the two *B. cinerea* isolates tested, i.e., 240 and 263 ppm. The IC50 values were in keeping with that of 149 ppm published by Olea *et al*. (2019) under slightly different conditions (different plant pathogen strains and eugenol directly incorporated into the same malt-agar medium). However, another eugenol IC50 value, much lower, of 39 ppm was published (Wang *et al*., 2010) under quite different experimental conditions, i.e., by assessing dry mycelium, incorporating eugenol into a different growth medium (PDB), and using a suboptimal incubation temperature for the plant pathogen, 25 °C. Similarly, Amiri *et al*. (2008) showed 75 % mycelial inhibition with 1000 ppm eugenol, which corresponded to approx. 4000 ppm under our conditions (our equation). This important difference could also be explained methodologically because Amiri *et al*. (2008) assessed mycelial growth inhibition after a long incubation period of 21 days, which seems quite long for a fast-growing fungus such as *B. cinerea*.

For the volatile mode of action of eugenol tested solely, with a specific and adapted *in vitro* methodology, different IC values for mycelial growth were obtained depending upon the plant pathogen isolate. For example, from our equations, 77 % inhibition corresponded to approx. 7080 ppm for isolate "P" and 293,090 ppm for strain "j". Based on 77 % inhibition with 50 ppm of volatile eugenol (Amiri *et al*., 2008), the authors found a much lower concentration that may be explained by the complex composition of the essential oils they tested, while we applied the pure single compound.

Furthermore, the higher efficiency of the vapour phase of essential oil compounds than the nonvolatile direct action against the plant pathogen *Aspergillus flavus* has been highlighted (Amiri *et al*., 2020; Velázquez-Nuñez *et al*., 2013). Our results confirmed this finding since, notably for isolate P, the linear regressions were similar for the vapour effect of eugenol and its double effect ("direct" also including a vapour effect) (Figure 1A,B).

For the eugenol anti-*B. cinerea* effect at relatively low dosages, the IC10 values found for our two isolates were consistent and similar, between 2.9 and 4.7 ppm, regardless of the mode of action of eugenol considered. Very interestingly, such concentrations corresponded to the level of total eugenol found in the berry skin of Baco blanc (Figures 3 and 4). Further studies may be necessary for testing such IC10 values on other fungal stages/organs (*e.g*., germination) to further confirm the significant partial antifungal activity that could be clearly put forward as a possible contribution of endogenous eugenol in the Baco blanc tolerance against *B. cinerea* as hypothesised beforehand in this study.

Furthermore, a clear isolate effect with a different sensitivity to eugenol was shown. Strain "j" was less sensitive than isolate "P", but only when the volatile mode of action of eugenol was activated. Thus, the existence of the dual action of this compound, direct and volatile antibiosis, was

confirmed but also showed the prime importance of isolate sensitivity, which must be considered to correctly interpret the overall effect on the plant pathogen. More fundamentally, this may arise from differences between isolates associated with the cellular transporter of eugenol well identified in *B. cinerea* cells. This transporter of lipophilic molecules, such as eugenol, is regulated by the *BcatrB* gene, which also interferes with the mycelial accumulation of the anti-*Botrytis* synthetic fungicide fludioxonil (substrate of BcatrB) (Schoonbeek *et al*., 2003). This fungal mechanism is of prime importance for a highly fungitoxic compound such as eugenol known for altering the fungal membrane structure, permeability and fluidity due to its lipophilic property and its accumulation in the phospholipid bilayer (Amiri *et al*., 2008; In't Veld *et al*., 1992; Olea *et al*., 2019; Sikkema, 1993; Sikkema *et al*., 1992). In addition, eugenol impairs the transportation of key biomolecules (amino acids and glucose) and increases oxidative stress by producing reactive oxygen species (ROS) (Zhao *et al*., 2021). The differential isolate sensitivity is also to be taken into account since some biocontrol products against *B. cinerea* are partly based on this compound (e.g., Mevalone®, Eden Research PLC, Oxfordshire, UK) (Fedele *et al*., 2020).

2. Partial tolerance of Baco blanc to *B. cinerea* compared to *V. vinifera* cultivars

In the vineyard, Baco blanc was confirmed as significantly resistant to *B. cinerea,* and this was already well noticed by François Baco himself (Baco, 1925; Baco, 1926) as well as more recently (Galet, 2015). The tolerance to the plant pathogen—or susceptibility—of a grapevine variety is modulated partly by bunch compactness, as reviewed by Tello and Ibáñez (Tello and Ibáñez, 2018) and often put forward (Vail and Marois, 1991; Vail *et al*., 1998; Alonso-Villaverde *et al*., 2008; Hed *et al*., 2009). In this study, our field measurements of Baco blanc bunch compactness showed average values (grade of 3 ± 0.5), indicating a perfect intermediate level in terms of compactness (Table 3 in the supplementary data). Therefore, this trait may not be very important in contributing to the high tolerance level of the hybrid cultivar. Another key factor of cultivar susceptibility to *B. cinerea* is the fruit maturity level, which will be discussed in detail below because it constitutes the fundamental grounds of ontogenic resistance to the plant pathogen in grapevine (Deytieux-Belleau *et al*., 2009; Kretschmer *et al*., 2007; Pañitrur-De La Fuente *et al*., 2018).

In this study, the intrinsic tolerance of Baco blanc berries (on a berry basis) to *B. cinerea* compared with two *V. vinifera* cultivars was also shown under our strict experimental conditions in a controlled biotest (Figure 2A). This allows us to postulate an explanation for tolerance possibly based on a particular chemical composition of the Baco blanc fruit. The Baco blanc berries from our two different vineyard plots were the most resistant with the lowest BBR severity (21.6 % and 38.3 %), compared with the Ugni Blanc berries expressing a BBR severity of 42.3 %, with the most highly susceptible being cv., Folle Blanche (87.7 %). The high susceptibility of the Folle Blanche cv is well known and, in the vineyard, potentially explained by very compact bunches (Galet, 2015), but here, we further demonstrate the intrinsic high susceptibility at the individual berry scale. The resistance of Baco blanc is also demonstrated by the reduction in sporulating symptoms (Figure 2B): the most resistant berries of Baco blanc (from the CONS32 plot) exhibited 9.0 % sporulation severity, whereas the Folle Blanche berries were the most sporulating (48.1 %). Furthermore, we have demonstrated, for the first time, that there was also significant variability in tolerance to *B. cinerea* within Baco blanc cv., that is, between different clones (Figure 5). *B. cinerea* susceptibility differences between grape clones of *V. vinifera* related to their cluster morphology were also demonstrated by (Alonso-Villaverde *et al*., 2008; Vail and Marois, 1991). Our controlled biotests on individual berries showed a significant clone effect in interaction with the type of symptom considered. This demonstrated, at the berry scale, the significant clone effect in interaction with the type of symptom considered. First, considering rot symptoms, E22 was the most susceptible clone, E12 was the most resistant clone, and c1086 was intermediate (Figure 5A,B). However, an opposite pattern was observed based on the sporulation severity at the berry surface: E22 was the most resistant clone, E12 was the most susceptible clone, and c1086 was intermediate (Figure 5C,D).

Interestingly, the eugenol content in the berry skin was related to these variations and could be proposed as a biochemical indicator of the ontogenic resistance of the Baco blanc fruit (Figure 4). The peculiar presence of eugenol in Baco blanc berry was recently discovered by Franc *et al*. (2023). Moreover, eugenol is highly concentrated in Baco blanc berries, 6 to 45 times more than in the two *V. vinifera* cultivars, Folle Blanche and Ugni Blanc (Franc *et al*., 2023). For comparison, our most concentrated Baco blanc skin sample is from clone E22, with 2580 $\mu g \times kg^{-1}$ of free eugenol, and was the most rotted and the least sporulating. Conversely, the skin sample of the E12 clone, which was opposed in susceptibility, was the least concentrated in free eugenol (1039 $\mu g \times kg^{-1}$). The clone c1086 was always intermediate (free-eugenol skin content at 1445 $\mu g \times kg^{-1}$). Thus, the greater the berry skin of a Baco blanc clone was concentrated in free eugenol, the lower the *B. cinerea* sporulation intensity expressed at the berry surface. Therefore, a possible anti-sporulating effect of free eugenol may be hypothesised. Furthermore, this relationship was independent of some other skin resistance factors (Table 2), notably the content of tannins, which are key compounds involved in the ontogenic resistance of the grapevine berry (Goetz *et al*., 1999; Pezet *et al*., 2003; Deytieux-Belleau *et al*., 2009). In the hybrid cultivar, the tannin concentrations (Table 2) were comparable to those of *Vitis vinifera* cultivars at the same fruit stage (Deytieux-Belleau *et al*., 2009; Pañitrur-De la Fuente *et al*., 2020). However, the relationship between the eugenol content and the maturity level of the fruit was clearly established, very importantly, since the total eugenol concentration was highly significantly correlated with the fruit TMI (Figure 6). Therefore, the riper the berries, the less concentrated eugenol is in the skinned fruit. Thus, the significant differences between clones in the free eugenol

concentration may be proposed as an original biomarker to better account for differences in susceptibility to *B. cinerea* within the Baco blanc cultivar. However, the free eugenol contents in the clones (Figure 4) were not sufficient, according to our IC50 calculations (Figure 1), to markedly inhibit the plant pathogen *B. cinerea*. An early resistance of the berries, due to a concentration of free eugenol but also to the presence of another form of this compound, can then be hypothesised. This hypothesis supports the idea of eugenol as a biochemical marker of ontogenic resistance in the Baco blanc berry.

3. Eugenol: an inducible molecule and a biochemical marker of Baco blanc ontogenic resistance

Among the major findings in these experiments, for the first time, the time-progress of eugenol content in the berry skin was finely quantified according to Baco blanc fruit development (Figure 3A). Interestingly, regardless of grapevine canopy management with or without leaf removal, total eugenol accumulated to reach a maximum at the véraison stage (at 719 GDD) and then decreased markedly during grape maturation. Such a time-progress eugenol pattern was different from that of pellicular tannins, which have been shown to decrease only very slightly after véraison (Deytieux-Belleau *et al*., 2009).

As another original finding, leaf removal at bunch closure in the Baco blanc canopy (377 GDD) induced a significant increase in eugenol production in the berry skin of 32.2 % at véraison and 38.6 % at harvest (1200 GDD) compared to control vines (Figure 3A). Therefore, eugenol in grapes was an inducible molecule possibly following abiotic stress, such as leaf removal. Similarly, skin tannins have also been shown to be induced by leaf removal (Pañitrur-De la Fuente *et al*., 2020). Defoliation causes less vigorous vines and more sun-exposed bunches, and induction could result from heat stress and/or sun radiation exposure (Keller, 2020). Moreover, the leaf removal tested under the Armagnac conditions resulted in a well-known and significant epidemiological effect by decreasing *B. cinerea* infection (Figure 7), as also often reviewed (Elmer and Reglinski, 2006; Latorre *et al*., 2015). For example, in our study, bunch zone defoliation may cause a decrease of 19.5 % in *B. cinerea* incidence and 13 % in BBR severity. Therefore, in this study, the consequences of leaf removal in the Armagnac vineyard may have also been caused by changing the bunch microclimate, increasing air circulation and sun irradiation within the bunch zone (Fermaud *et al*., 2001; Pieri and Fermaud, 2005; Keller, 2020).

The eugenol-bound molecular form was induced significantly by leaf removal at véraison, and at harvest, leaf removal resulted in a significant increase in the free eugenol concentration (Figure 3B). A recently published protocol allowed us to differentiate the two molecular forms of eugenol, the free form and a so-called bound form corresponding to unidentified eugenol precursors that are enzymatically released (Franc *et al*., 2023). By considering eugenol and related compounds as defence molecules, it may be hypothesised that the eugenol-bound forms, i.e., possibly glycosidic forms, could be activated by β-glucosidase exoenzymes produced by *B. cinerea* during infection, releasing then the fungitoxic form that is free eugenol (Le Roy *et al*., 2016; Sasaki and Nagayama, 1994). The bound eugenol forms could be glycosidic precursors, and studies are underway in the laboratory to identify these eugenol precursors in Baco blanc. For other glycoconjugate compounds in other plants, notably flavonol glycosides, it has been shown that UV stress increased the concentrations of rhamnosylated kaempferol and quercetin glycosides in *Arabidopsis thaliana*. (Hectors *et al*., 2014). Such an accumulation of the bound form following abiotic stress may also be put forward in the Baco blanc case and would constitute a reserve of aglycone that could be easily mobilised to face later stress (Gachon *et al*., 2005; Vogt and Jones, 2000). Finally, the increased free eugenol content at harvest due to leaf removal may be interpreted as an expected subsequent transformation of the bound form, considered precursors, into the free form in the skin berry tissues during the maturation process.

CONCLUSIONS

This original work on Baco blanc, cultivated in Armagnac as the only hybrid variety authorised in French PDO, allowed us to better understand some mechanisms involving endogenous eugenol, accounting for cultivar resistance to major grapevine pathogens, notably *B. cinerea*. Therefore, further studies will be of interest by addressing similar questions with other major pathogens, such as *Plasmopara viticola* and/or *Erysiphe necator*. To reduce the use of synthetic pesticides in vineyards, this study will also be helpful by focusing on and further investigating the eugenol molecule in its different biochemical forms, which could provide or be part of biocontrol products against major grapevine pathogens. The efficiency of eugenol against *B. cinerea*, with an IC50 comparable to some other values in the literature, is clearly based on two modes of action, including a powerful one that is the vapour inhibiting effect. The eugenol concentrations found in the berry skin of Baco blanc at harvest corresponded more to IC10 values, i.e., at relatively low but active concentrations. Baco blanc resistance to the plant pathogen was further confirmed by comparison with *V. vinifera* cultivars also used in the Armagnac region: Folle Blanche and Ugni Blanc. In addition, the significant intravarietal variability between Baco blanc clones, as well as the temporal study of the two forms of eugenol during grape berry development, allowed us to propose eugenol as a key biochemical marker of both ontogenic and varietal resistance of Baco blanc to *B. cinerea*. Finally, this study also demonstrated, for the first time, the inducibility of this molecule following leaf removal. Therefore, such inducibility may play a part in the anti-Botrytis epidemiological effect of this cultural technique in this specific cultivar case. However, there are still several questions that remain open, such as i) the supposedly better efficacy against *B. cinerea* of the bound form of eugenol, ii) the nature of the eugenol precursors, and iii) the status and

stability of the clones within this historical, one-century-old hybrid variety of great viticultural interest.

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