Research Article

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Antifungal activity of chili pepper extract with potential for the control of some major pathogens in grapevine

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Abstract

BACKGROUND: In recent years, biofungicides have drawn increasing interest in vineyards for a more sustainable integrated and copper-limited pest management. Among alternatives, botanicals could represent valuable tools, being rich sources of biologically active compounds. Conversely to the well-known antioxidant and biological properties in relation to health benefits, investigation on bioactivity of hot pungent *Capsicum* sp. products against fungal phytopathogens in vineyards is still scarce. Therefore, the present study aimed at exploring the biologically active compounds profile of a chili pepper (*Capsicum chinense* Jacq.) pod extract and its antimicrobial properties against some of the major fungal and Oomycetes pathogens of grapevine, including *Botrytis cinerea* Pers., *Guignardia bidwellii* (Ellis) Viala & Ravaz and *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni.

RESULTS: The ethyl acetate-extracted oleoresin from the most pungent varieties was rich in capsaicinoids and polyphenols (371.09 and 268.5 μg mg^{-1} dry weight, respectively). Capsaicin and dihydrocapsaicin, hydroxycinnamic and hydroxybenzoic acids and quercetin derivatives were the most abundant, while carotenoids represented only a minor fraction. The oleoresin was efficient to inhibit all three pathogenic fungi and ED_{50} values were determined, evidencing that *G. bidwellii* was the more sensitive (0.233 ± 0.034 mg mL^{-1}).

CONCLUSION: The results suggested a potentiality of chili pepper extract for the control of some important grapevine pathogens, their possible application being helpful for the recommended limitation in extensive use of copper in vineyard. The complex mixture of high amounts of capsaicinoids, associated to specific phenolic acids and other minor bioactive components might contribute to the observed antimicrobial action of chili pepper extract.

Supporting information may be found in the online version of this article.

Keywords: biological control; *Botrytis cinerea*; *Capsicum* spp.; grapevine; *Guignardia bidwellii*; *Plasmopara viticola*

1 INTRODUCTION

In relation to the cultivated area (7.5 billion ha)[1] and economic value, grapevine (*Vitis vinifera* L.) is one of the most important fruit crops worldwide. Due to the wide range of pathogens affecting grapevine, causing direct and indirect damage to the production, an intense use of pesticides is required for the protection of this crop.[2,3] Together with *Erysiphe necator* (causal agent of powdery mildew), *Botrytis cinerea* Pers. and *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, respectively the causal agents of gray mold and downy mildew, are the pathogens for which most treatments are required.[2,4]

Copper-based pesticides represent one of the main means of containment of gray mold and downy mildew, especially in organic viticulture where its use is still authorized due to its wide range of action.[5,6] However, the extensive use of copper can lead to issues at different scales. From an ecological point of view, copper accumulated in the soil affects plants and fauna.[6,7] Moreover, the presence of copper and other fungicides residues in grapes and wine can be a threat to human health.[7]

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In this context, the European Union has been regulating for years to reduce and/or replace the use of copper. Currently, according to EU regulation 2018/1981[8] the maximum amount of copper allowed is 28 kg ha^{-1} over a period of 7 years (i.e., 4 kg ha^{-1} per year). Moreover, in the Farm to Fork (F2F) strategy, the EU targets 25% of organic land by 2030 and also to reduce by 50% the use of chemical pesticides.[9]

One of the strategies to reduce or replace copper and other synthetic pesticides use is the development of grapevine resistant varieties, the use of which has recently been allowed by the EU Commission for the production of wines with protected denominations of origin.[3,10] In particular, in recent decades numerous efforts have primarily focused on breeding in order to obtain varieties resistant to downy and powdery mildew, and gray mold.[11] The use of these cultivars allows the reduction of fungicide applications. However, the onset of secondary diseases previously controlled by phytosanitary treatments occurs as a backlash.[3] *Guignardia bidwellii* (Ellis) Viala & Ravaz, the causal agent of black rot disease, has diffused from Northern America and increased in importance in the last few years in northern wine-growing regions in Europe, becoming one of the more serious new emergent grapevine pathogens in Italy and France, favored by both reduction of copper dosage allowed in organic viticulture and for the significant reduction of fungicide treatments on mildew resistant/tolerant varieties, yet black-rot susceptible, in integrated management.[3,12,13] The latter factors are favoring the increase of black rot outbreaks and leading to relevant yield loss in European viticultural production.[14,15]

The research on botanicals with bioactive action against phytopathogens has received increasing interest, since biopesticides could represent widely acceptable, sustainable as environment-friendly and potentially lower toxic substitutes to synthetic fungicides in combination with limited copper application in an integrated management program.[16,17] Recently, the use of the biocontrol agents has been highly endorsed by European Commission's recommendations in the Farm to Fork (F2F) strategy for sustainable food,[9] under the European Green Deal. Many traditional or novel aromatic and medicinal plants represent an important source of secondary metabolites such as alkaloids, tannins and flavonoids, quinones, glycosides, saponins and terpenoids, conferring a huge range of variable antimicrobial properties.[7,16,18–20] These bioactive molecules are naturally accumulated as plant defense barriers against phytopathogens[21] and represent strategies for environmental adaptation to abiotic stresses[22] (*e.g.*, UV exposure, dehydration, high temperatures), or as pollinator's attractants.[23] Among the most domesticated crops utilized for centuries as culinary spice, chili peppers (*Capsicum* spp.) have been recognized to be rich in health-beneficial bioactive phytochemicals for the human diet and to possess important medicinal properties.[24–26] Regarding biochemical composition, the five most widely cultivated chili pepper species (*C. annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. baccatum* L. and *C. pubescens* Ruiz and Pav.) are recognized as important sources of vitamins and several bioactive compounds.[27,28] The most important are carotenoids and flavonoids (responsible for the attractive colors of fruits and functioning as natural antioxidants and defenses against biotic and abiotic stresses), and more of 20 capsaicinoids (simple arylalkylamide alkaloids), the principal member of which is capsaicin (*trans*-8-methyl-N-vanillyl-6-nonenamide), synthesized mainly in the placenta, conferring the typical pungent taste.[24,29] Among the latter, capsaicin and dihydrocapsaicin are the most abundant capsaicinoids found in chili peppers, being 69 and 22%, respectively, followed by nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin, in a wide range of variance affected by fruit ripening, species, cultivars, and cultivation conditions.[29,30] Most of these bioactive compounds are already known to exert antimicrobial or antifungal actions both as single molecules or as major components of pepper extracts and oleoresins.[31–37] A number of reports confirmed also that *Capsicum* sp. extracts, in addition to be proposed as biopesticide against insects,[38] were effective against plant pathogens as post-harvest fungal agent in apple[31] and several agriculture fungi like *Alternaria alternata*, *Fusarium oxysporum*, *Botrytis cinerea*, *Verticillium dahliae*, *Phytophtora capsici*.[39–43] Also, it has suggested that capsaicinoids accumulation in wild chili peppers may represent an adaptive response to *Fusarium* seed infection in an evolutionary sense.[40] In some cases, along with an antifungal direct action, some secondary metabolites found in chili peppers were also proven to elicit natural plant induced resistance.[41,43–45]

Despite the recognized inhibitory actions against several pathogenic fungi of plant crops, to date investigation on antifungal activity of hot pungent *Capsicum* sp.-based products against the most important pathogens of *Vitis vinifera* is still scarce, with the exception of one existing published patent about aqueous solution comprising *Capsicum* for contrasting downy or powdery mildew.[46]

Accordingly, in this research, we examined the potential grapevine fungicide activity of a chili pepper oleoresin, obtained from fruits of the hottest varieties of *C. chinense* and extracted through maceration with a safe and eco-friendly solvent. The profile of the major bioactive compounds was also discussed in relation to their putative action against some of the most detrimental phytopathogens in viticulture production.

2 MATERIALS AND METHODS

2.1 Chemicals

Capsorubin was obtained from CaroteNature Gmbh (Musingen, Switzerland). Capsaicin (99.9% purity), capsanthin, zeaxanthin, β-cryptoxanthin, potato dextrose agar (PDA), β-carotene, lutein, β-Apo-8-carotenal (*trans*), dichloromethane and ethyl acetate were purchased from Merck, Darmstadt (Germany); violaxanthin, anteraxanthin and flavonoids from Extrasynthese, Genais (France); ethanol (analytical grade) from Carlo Erba Reagents, Milan (Italy); acetonitrile and absolute methanol from Thermo Fisher Scientific (Illkirch, France). Fluorescein, 2,2′-azobis (2- methylpropionamidine) dihydrochloride (AAPH) and hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), were purchased from Sigma-Aldrich (France). The water used for HPLC–MS was purified with an Elga system (Bucks, U.K.). Formic acid was obtained from Sigma Chemical Company (St Louis, MO). Phenolic acids were purchased from Sigma.

2.2 Preparation of chili pepper oleoresin

Commercial dry powder of fruits and seeds of *Capsicum chinense* Jacq., var. 'Habanero Mustard', 'Habanero Pastel', 'Trinidad Moruga Scorpion Red', 'Trinidad Moruga Scorpion Choco', 'Carolina Reaper', 'White Naga', 'Naga Morich Chocolate' was purchased by means of local producers (Italy). The plant material was chosen on the basis of the highest pungency level [ranging from 350 000 Scoville heat units (SHU) up to more than 1 million SHU],[47–49] a parameter which is known to be directly related to capsaicinoid content. Since the different chili pepper powders were obtained from plant material available on the internet market, we prefer

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to be not strictly dependent on characteristics of a single variety. Indeed, this could be a limit to an easy and quick acquisition of the plant material and so we prefer to make a blend that represented an intermediate option, easy to be replicated.

In the case of all varieties used, the percentage of the powders added to the total mixture was always the same and the oleoresin was extracted by macerating them in 100% ethyl acetate (100 g L^{-1}) for 2 h at 25 °C under stirring. After centrifugation at 28 000 × *g* for 10 min (Beckman Coulter, Avanti J-26S XP, Milan, Italy), the supernatant fraction was collected, and the solvent removed by rotary evaporator (Buchi, Rotavapor® R-100, Cornaredo, Italy). The final extract was diluted in 100% ethanol obtaining a stock solution of approx. 400–500 mg dry weight (DW) mL^{-1}, stored at −20 °C until use.

2.3 GC–MS analysis of chili pepper oleoresin

Oleoresin extract composition was determined by GC–MS (Agilent Technologies GC–MS 5977E, USA) and the amount of each compound was expressed as percentage (area percent method), and also in μg mg^{-1} DW by external calibration with capsaicin standard.

Oleoresin extract was dried under nitrogen and resuspended in dichloromethane (Merck, Darmstadt, Germany)[50] leading to a solution of known concentration; the sample was transferred in vial suitable for injection. GC–MS was performed in EI mode (70 eV) with a 5977E MSD system, a single-quadrupole (Agilent Technologies, USA) equipped with 7683A autosampler and automatic split/splitless injector. An aliquot (1 μL) of solutions was injected in splitless mode for the first 1.5 min of the analysis and then in the split mode for the remainder of the run. The separation was carried out on SP-5 ms capillary column (30 m × 0.25 mm × 0.25 μm) (Supelco, Bellefonte, PA). The GC oven program started for 10 min at 60 °C; then, the temperature was ramped up (3 °C min^{-1}) to 95 °C (hold time 5 min), then again to 270 °C at the rate of 3 °C min^{-1} and to end with 5 min hold time. The total run time was 89.93 min and in post run the temperature was set to 280 °C for 5 min in order to clean the column. The length of the run was necessary to check up to which minute compounds of interest eluted; after 79 min, however, nothing of interest was detected. Helium (6.0) was used as carrier gas with a constant flow rate of 1.0 mL min^{-1}. The temperature value of the ion source and the quadrupole were set to 240 °C and 150 °C, respectively; the transfer line was set to 240 °C. The GC–MS analyses in the full scan mode for a range of 50 to 650 amu and the analysis were performed after a solvent delay of 4 min, with 3 microscan sec^{-1}. Compounds were identified by comparing their mass spectra of NIST 14 Mass spectral Library and by matching the results with those reported in the literature.[50–52]

2.4 HPLC analysis of principal polyphenols

To 50 mg of a mixture of a fine powder from chili pepper pods, prepared as the one used to obtain oleoresin, 1 mL of 70% methanol was added and the mixture was put into an ultrasonic water bath at 25 °C for 15 min. This extraction step was based on the one developed by Jeong *et al.*[53] for polyphenols extraction of pepper. After centrifugation (15 000 rpm, 5 min), the supernatant was collected and placed in a new tube. The powder was extracted a second time in a similar manner. All the supernatants were combined and diluted with water (1:1 v/v) and newly centrifuged previous HPLC-MS injection.

Analysis of polyphenols was performed by HPLC-MS/MS in Multiple Reaction Monitoring (MRM) using a 1260 Infinity UPLC (Agilent Technologies, Courtaboeuf, France) coupled to a 6430 triple quadrupole mass spectrometer (Agilent Technologies, Courtaboeuf, France) and adapting a previous protocol.[54] Two μL of sample were injected into an Agilent Zorbax SB-C18 (100 mm × 2.1 mm, 1.8 μm) thermostated at 40 °C, and separation of the compounds was performed at a flow rate of 0.43 mL min^{-1} with a mobile phase composed of solvent A (distilled water, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid). The gradient was as follows: 0 to 4 min, (1% B-10% B); 4 to 12 min (10% B 8% to 20% B); 12 to 13 min (20% B to 30% B); 13 to 16 min 30% B; 16 to 20 min (30% B to 50% B); 21 to 25 min, 95% B. The source parameters were: capillary voltage, +3000 V; nebulizer pressure, 15 psi; dry gas, 11 L min^{-1}; dry temperature, 350 °C. Specific MRM transitions for each polyphenol were used for identification[54] and quantification was done with the Mass Hunter Data Analysis software (Agilent, Technologies, Courtaboeuf, France). Polyphenols were determined from calibration curves of pure standards (injected concentrations ranging from 0.08 to 43 μg mL^{-1}). Feruloyl hexoside compounds were tentatively identified by their MRM fragmentation and quantified as equivalent of kaempferol hexoside. Concentrations were expressed in μg mg^{-1} DW of phenolic compound.

2.5 HPLC analysis of principal carotenoids

Carotenoids such as capsorubin, violaxanthin, capsanthin, anteraxanthin, zeaxanthin, *β*-criptoxanthin, *β*-carotene and lutein were extracted according to Su *et al.*[55] and to Borguini *et al.*[56] with some modifications. Briefly, 20 μL of oleoresin and 100 μL of internal standard (IS) *β*-Apo-8-carotenal (*trans*) (65 mg L^{-1}) were mixed in a Falcon tube, then ethyl acetate (1 mL) *and* Milli-Q® water (2 mL) were added and the sample was centrifuged at 3000 rpm for 15 min on a Centrifuge 5415 (Eppendorf AG, Germany). Sodium sulfate anhydrous was added to dry and the sample was kept in the dark for about 20 min, then the organic phase was filtered through syringe filter (PTFE 0.22 μm, 25 mm, DTO Servizi Srl, Venice, Italy). Two hundred milliliters of extract were then evaporated to dryness under nitrogen, reconstituted with 200 μL of acetone and filtered through syringe filter. All samples were stored at −20 °C until analysis. The filtrate was transferred into an autosampler vial and finally 20 μL were injected into HPLC. The HPLC system included a Shimadzu LC-20AT pump, a vacuum degasser, a Prominence SPD-M20A photodiode-array detector, a Prominence SIL-20 AC HT autosampler (20 μL loop) and a Prominence CTO-20 AC column oven set at 25 °C (Shimadzu Corporation, Kyoto, Japan). The HPLC separation was achieved using a Spherisorb column ODS 2 (4.6 × 250 mm, 5.0 μm particle size) and a mobile phase of acetonitrile: methanol (40:60) at a flow rate of 1 mL min^{-1} was used. The optimal wavelength for the detection of the main carotenoid components in the extract was found to be 454 nm and the detector slit width was 4 nm. Full spectra were recorded in the range 190–800 nm. Equipment control, data acquisition and integration were performed with Shimadzu LabSolutions (Ver. 5.54 SP2) Software.

2.6 Total carotenoid quantification by spectrophotometric analysis

The average carotenoid quantification of the dried oleoresin extract was determined according to the spectrophotometric method of Biehler *et al.*[57] Dry extract was suspended in 0.5 mL acetone and sonicated for 2 min. The absorbance at the mean absorbance maximum (A_{450}) of increasing dilutions of the suspension was read in a 1 mL-quartz cuvette by spectrophotometer

(Agilent Technologies, 8453, Milan, Italy). The average carotenoid concentration (c) was calculated by the equation:

$$c\left(\text{mol L}^{-1}\right) = A_{450}\text{*dilution factor}/135310$$

and expressed as mg per 100 g of oleoresin by using an average molar mass of 536.88.

2.7 Antioxidant potential assay

The antioxidant capacity of the oleoresin extract was evaluated with the Oxygen Radical Absorbance Capacity (ORAC) method. The assay was made according to a modified method developed by Dávalos *et al.*[58] by using an automated plate reader (Fluostar Omega; BMG Labtech, Offenburg, Germany). Briefly, oleoresin (20 µL), Trolox (standard) or phosphate buffer (blank) prepared in 75 mM phosphate buffer (pH 7.4) and 120 µL of fluorescein solution (70 nM final concentration) were mixed with in a 96-well black plate, then placed at 37 °C during 5 min. After that, 60 µL of AAPH (12 mM final concentration) were added and the reaction was immediately followed by monitoring fluorescence using 485 nm excitation and 530 nm emission wavelengths at each cycle of 90 min. A standard curve of Trolox was used and its ORAC value was obtained by using the Trolox standard curve $y = 44.231.47x + 140.977.55$ with a $R^2 = 0.89$. The antioxidant activity of the pepper extract was expressed as µmoles Trolox equivalents (TE) g^{-1} DW extract (ORAC). All samples were analyzed five times.

2.8 Pathogen *inoculum* source

B. cinerea Pers. isolate used in the experiment was isolated from harvested bunches in local vineyards (Udine, Italy) since 2018 and grown on potato dextrose agar (PDA) medium at 21 °C under a 12 h photoperiod; the isolate was multiplied every 2 weeks by hyphal plug or exposed to actinic blue light under a 12 h photoperiod during one more week for inducing sporulation. Conidial suspension was adjusted at the concentration of 4×10^5 conidia mL^{-1} in sterile distilled water, before inoculation on PDA for the growth inhibition assessment.

G. bidwellii (Ellis) Viala & Ravaz isolate was obtained in June 2018 under sterile condition, by selecting single pycnidia from symptomatic leaves of infected grapevines, at the University of Udine. The strain was grown and maintained on PDA at 21 °C under a photoperiod of 12 h light and transferred in fresh medium every 3–4 weeks for maintenance. A plug of 3–4-week-old mycelium (5 mm Ø) was aseptically removed and used as fungal inoculation for growth inhibition assessment.

P. viticola (Berk. & M.A. Curtis) Berl. & De Toni sporangia were collected from natural infected leaves in an untreated vineyard in 2019 (University of Udine, Italy) and weekly propagated by infecting 4th or 5th leaf of one-year old potted vines of *Vitis vinifera* cv. Sauvignon Blanc grafted on SO4 rootstock and maintained in controlled conditions at 25 °C under a 12 h photoperiod, 55% of relative humidity. The fresh sporulation was collected the day of the experiment, counted by microscope under visible light and adjusted with sterile distilled water at the final concentration of 4×10^5 sporangia mL^{-1}.

All pathogen isolates were identified on the basis of disease symptoms on plants where they were collected and on microscope observation of characteristic morphological traits of reproductive structures in artificial substrate and/or host surface.

2.9 *In vitro* antifungal activity against *Botrytis cinerea*

The inhibition of different concentrations of chili pepper oleoresin on *B. cinerea* growth was analyzed by the diffusion technique on PDA growth medium. Eleven doses of oleoresin (0.44–8.86 mg mL^{-1} in sterile distilled water with 4.9% (v/v) ethanol) were prepared for the assay and the diluted solutions (70 µL) were spread on 5 mL of solidified PDA medium in Petri dishes (50 mm Ø) just before inoculation with a drop of 5 µL of conidial suspension, adjusted at 4×10^5 conidia mL^{-1}. Sealed Petri dishes were incubated in a growth chamber at 21 °C under a 12 h photoperiod, until full evasion of fungus was reached in control samples. The mycelium growth area was monitored daily and photographs of Petri dishes with mycelia were taken and images analyzed by ImageJ Fiji software[59] for the assessment of percentage of growth inhibition with respect to the control. Three independent sets of experiments were performed with four replicates each set.

2.10 *In vitro* antifungal activity against *Guignardia bidwellii*

Inhibition activity of different concentrations of chili pepper oleoresin against *G. bidwellii* was determined by aseptically incubating a plug of 3–4 week-old mycelium (5 mm Ø) on 3 mL of PDA medium in 6 well cell culture microplates for growth test assessment. The diluted solutions (70 µL at 0.001 to 12.5 mg mL^{-1} in sterile distilled water with 6.9% (v/v) ethanol) were spread on PDA medium just before inoculation. Radial growth of the mycelium was monitored for 3, 6, 9 and 12 days, respectively and weighted to each respective area of the plug at time 0. Photographs of Petri dishes with mycelia were taken and images were then analyzed by ImageJ software for the assessment of the growth area. Three independent sets of experiments were performed with four replicates each set.

2.11 Inhibition of *Plasmopara viticola* sporulation on leaf discs

The inhibitory effect of different concentrations of chili pepper oleoresin extract against *P. viticola* sporulation was analyzed on leaf discs, according to Corio-Costet *et al.*[60] Eleven doses of oleoresin (0.2–10 mg mL^{-1}) in sterile distilled water with 3.8% (v/v) ethanol were prepared for the assay the day of experiment. Four leaf discs (21 mm Ø) on wet Whatman filter paper were sprayed with each solution (1.25 mL) and incubated for 1 d in the dark before inoculation with three drops (15 µL) of fresh *P. viticola* inoculum adjusted at 4×10^5 sporangia mL^{-1}. Sealed Petri dishes were incubated in a humid chamber at 21 °C under a 12 h photoperiod until complete evasion of conidiophores. Photographs of Petri dishes with sporangia evasion were taken after 5 days, and images were analyzed by ImageJ software for the assessment of percentage of sporulation to total leaf area, as described in Peressotti *et al.*[61] Three independent experiments were performed with four replicates each set.

2.12 Statistical analysis

Antifungal activity of chili pepper oleoresin was statistically analyzed using R extension package drc[62] and for each pathogen a regression curve was fitted using dose–response analysis sigmoidal model Log-logistic. The effective dose or concentration (ED_{50}) of oleoresin to inhibit 50% of fungal growth was derived by log-logistic dose–response curve analysis.[63]

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Table 1. GC–MS analysis of chili pepper extract

Name of compound	Formula	%	m/z	Content (μg mg^{-1} DW)	Biological activity
Undecane	$C_{11}H_{24}$	1.067			Antioxidant[66,67]
Butanoic acid, 3-methyl-, hexyl ester	$C_{11}H_{22}O_2$	0.054			
cis-Muurola-3,5-diene	$C_{15}H_{24}$	0.017			
5-Octen-1-ol, (Z)-	$C_8H_{16}O$	0.078			
1,3-Pinanylamine	$C_{10}H_{19}N$	0.007			
3,7-Diacetamido-7H-s-triazolo[5,1-c]-s-triazole	$C_7H_9N_7O_2$	0.005			
N-[4-Aminobutyl]aziridine	$C_6H_{14}N_2$	0.007			
α-Himachalene	$C_{15}H_{24}$	0.103			
2,6,6-Trimethyl-bicyclo[3.1.1]hept-3-ylamine	$C_{10}H_{19}N$	0.016			
2-Methylbutyl 8-methylnon-6-enoate	$C_{15}H_{28}O_2$	0.113			
Z,Z-2,5-Pentadecadien-1-ol	$C_{15}H_{28}O$	0.098			
2-Trifluoroacetoxydodecane	$C_{14}H_{25}F_3O_2$	0.102			Antimicrobial[68,69]
8,9,9,10,10,11-Hexafluoro-4,4-dimethyl-3,5-dioxatetracyclo[5.4.1.0(2,6)0.0(8,11)]dodecane	$C_{12}H_{12}F_6O_2$	0.006			Antimicrobial[70]
13-Tetradecenal	$C_{14}H_{26}O$	0.026			
10-(3-Ethyl-2-oxiranyl)-1-decanol	$C_{14}H_{28}O_2$	0.036			
Imidazole, 2-amino-5-[(2-carboxy)vinyl]-	$C_6H_7N_3O_2$	0.033			Antimicrobial[71]
6-Tridecene, (Z)-	$C_{13}H_{26}$	0.044			
Hexadecen-1-ol, trans-9-	$C_{16}H_{32}O$	0.139			Antioxidant[72]
γ-Guanidinobutyric acid	$C_5H_{11}N_3O_2$	0.010			
unknown		0.012			
1-Octanesulfonyl chloride	$C_8H_{17}ClO_2S$	0.012			
Cyclopentanone, 2-(1-methylpropyl)-	$C_9H_{16}O$	0.013			
Z-2-Dodecenol	$C_{12}H_{24}O$	0.011			
l-Guanidinosuccinimide	$C_5H_7N_3O_2$	0.003			
E-2-Tetradecen-1-ol	$C_{14}H_{28}O$	0.358			
(Z)6-Pentadecen-1-ol	$C_{15}H_{30}O$	0.038			Antimicrobial[73]
(8Z)-14-Methyl-8-hexadecen-1-ol	$C_{17}H_{34}O$	0.059			
2(1H)-Benzocyclooctenone, decahydro-10a-methyl-, trans-	$C_{13}H_{22}O$	0.092			
3-Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$	0.091			Antioxidant[74]
7-Hexadecenal, (Z)-	$C_{16}H_{30}O$	0.100			
Methyl 12,13-tetradecadienoate	$C_{15}H_{26}O_2$	0.021			
3-Trifluoroacetoxydodecane	$C_{14}H_{25}F_3O_2$	0.046			
10-Bromodecanoic acid, ethyl ester	$C_{12}H_{23}BrO_2$	0.012			
Octadecanal	$C_{18}H_{36}O$	0.139			Antimicrobial[75]
Nerolidol	$C_{15}H_{26}O$	0.134			Antioxidant/antibacterial/antifungal/insecticidal/anti-inflammatory/defense-related terpenes in *Vitis* and *Camellia*[76–81]
E-11-Hexadecenal	$C_{16}H_{30}O$	0.093			
1,10-Hexadecanediol	$C_{16}H_{34}O_2$	0.005			
unknown		0.015			
Methyl 8-methyl-nonanoate	$C_{11}H_{22}O_2$	0.008			
N-Pentadecylacetamide	$C_{17}H_{35}NO$	0.228			
9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	0.198			
γ-Thionodecalactone	$C_{10}H_{18}OS$	0.065			
N-Hexadecylacetamide	$C_{18}H_{37}NO$	0.095			
unknown		0.029			
9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	0.048			
1-Ethyldodecyl acrylate	$C_{17}H_{32}O_2$	0.022			
p-Cresol, 2,2′-methylenebis[6-tert-butyl-	$C_{23}H_{32}O_2$	0.037			
Pelargonic acid vanillylamide (Nonivamide)	$C_{17}H_{27}NO_3$	0.614	**137**, 151, 293	37.81	Antioxidant/antifungal[40,41,82]

Table 1. Continued

Name of compound	Formula	%	m/z	Content (μg mg^{-1} DW)	Biological activity
Tetradecane, 2,6,10-trimethyl-	C17H36	0.239			
Nordihydrocapsaicin	$C_{17}H_{27}NO_3$	0.373	**137**, 151, 293	37.53	Antioxidant/antifungal[40,41,82]
Dihydro-*β*-agarofuran	$C_{15}H_{26}O$	0.068			
Capsaicin	$C_{18}H_{27}NO_3$	69.436	**137**, 152, 305	118.58	Antioxidant/antifungal[40,41,82]
Dihydrocapsaicin	$C_{18}H_{29}NO_3$	23.755	**137**, 151, 307	64.97	Antioxidant/antifungal[40,41,82]
Dihydrocapsaicin, O-acetyl-	$C_{20}H_{31}NO_4$	0.675	**137**, 195, 307	37.88	Antioxidant/antifungal[40,41,82]
unidentified		0.080	137	37.09	
unidentified		0.122	137	37.23	
Heptacosane	$C_{27}H_{56}$	0.505			Antibacterial[83]
unidentified		0.029			
Octacosane	$C_{28}H_{58}$	0.206			Antibacterial[84]
Total Capsaicinoids				371.09 (37.11)*	

Note: Peak area relative amount ratio (%), based on the normalized value (100.0%) and content of the compounds detected from chili pepper oleoresin after chromatographic run in dichloromethane.
*Relative abundance (% wt) on oleoresin DW basis.

3 RESULTS

3.1 GC–MS analysis of chili pepper oleoresin

To investigate the antifungal efficacy of chili pepper extracts against some fungal pathogens in vineyard, a number of the hottest varieties belonging to the highly pungent species *C. chinense* were selected in relation to their high content in carotenoid and capsaicinoids. The choice of a mix of varieties instead of a single one was dictated by the fact that this option could be more easily reproduced from commercial market. Of course, if we would be able to produce by ourselves one of the best varieties this would be the best choice.

The powder mixture of each of these varieties was macerated in equal proportion in ethyl acetate, chosen as a very good organic solvent for achieving high capsaicinoid recovery in oleoresins[64,65] and GC–MS analysis was then performed for quantification and characterization of these compounds.

Comparing GC–MS chromatograms of pure capsaicin (Supporting Information, Fig. S1, panel A), used as standard, with that of oleoresin sample (Supporting Information, Fig. S1, panel B), it can be observed that its peak overlapped quite similarly with the corresponding one identified in the oleoresin sample, with a retention time of 74.096 and 74.218 min, respectively. Among the 79 detected compounds in the oleoresin under analysis, 48 were identified by matching their spectra with the NIST library and reported in Table 1, in relation to their relative peak area (as a percentage of the whole chromatogram) and their potential bioactivity. The benzyl cation fragment at m/z 137, commonly found in all capsaicinoids of pepper extracts, has been used for their specific identification in SIM mode of GC–MS analysis, along with m/z 305, 307, 293.[51]

Among capsaicinoids, the most abundant principal compounds in relation with their % of total peak area were represented by capsaicin and dihydrocapsaicin, and, at minor extent, *O*-acetyl dihydrocapsaicin, nonivamide and nordihydrocapsaicin, followed by two unidentified capsaicinoids. The results obtained also evidenced that capsaicin and dihydrocapsaicin contribute for the highest amount (69.44 and 23.76% of the total compounds analyzed by GC–MS, respectively) in chili pepper extract, whereas the other minor capsaicinoids (nonivamide and nordihydrocapsaicin) accounted only for low percentage (0.987%). As expected, total capsaicinoids were well represented, reaching approximately 95% of the total amount of various compounds detected by GC–MS analysis in oleoresin.

In term of abundance, other most represented compounds identified were alkanes (Undecane, Heptacosane and Octacosane, 1.07, 0.51 and 0.21% of the total, respectively), essential oils (*E*-2-Tetradecen-1-ol and Hexadecen-1-ol, trans-9-, 0.36 and 0.139%, respectively), terpenes (2,6,10-Trimethyltetradecane, Nerolidol, *α*-himachalene, 0.24, 0.13, and 0.10%), oleic acid derivative (9-Octadecenamide, (Z)-, 0.2%), and volatile fatty aldehydes (Octadecanal, 0.14%). Some minor components were acids (as 3-Methylbutanoic acid), alcohols (5-Octen-1-ol, (Z)-), aldehydes (*E*-11-Hexadecenal and 13-Tetradecenal), esters (as Butanoic acid, 3-methyl-, hexyl ester and 2-Methylbutyl 8-methylnon-6-enoate). By means of linear regression equation obtained from calibration with the internal standard of capsaicin, the concentration of the more abundant capsaicinoid found in oleoresin reached 118.58 μg mg^{-1} DW and total content of capsaicinoids accounted for the value of 371.09 μg mg^{-1} DW.

Table 2. Polyphenol concentration and biological activity in chili pepper extract

Compounds	Content (μg mg^{-1} DW)	Biological activity
Phenolic acids		
Hydroxycinnamic acids		
Caffeic acid	1.5 ± 0.1 (0.58)*	
Vanillic acid	174.0 ± 9.4 (64.91)*	Antifungal[85]
Ferulic acid	14.4 ± 0.6 (5.38)*	Antifungal[86]
Feruloyl hexoside isomer	1.4 ± 0.3 (0.52)*	
Feruloyl hexoside isomer	3.6 ± 0.2 (1.34)*	
Feruloyl hexoside isomer	1.3 ± 0.2 (0.48)*	
Feruloyl hexoside isomer	1.8 ± 0.2 (0.68)*	
Hydroxybenzoic acids		
Protocatechuic acid	35.8 ± 0.6 (13.35)*	Antifungal[87]
Flavonoids		
Flavonols		
Quercetin 3-glucuronide	0.5 ± 0.1 (0.20)*	
Quercetin 3-rhamnoside	13.2 ± 1.6 (4.94)*	Antifungal[88]
Quercetin 3-rutinoside	1.2 ± 0.4 (0.46)*	
Kaempferol 3-glucoside	<LOD	
Flavanones		
Naringenin	1.3 ± 0.1 (0.47)*	
Naringenin glucoside	16.0 ± 1.7 (5.98)*	Antifungal[89,90]
Flavanols		
Catechin	<LOD	
Epicatechin	<LOD	
B1	<LOD	
B2	<LOD	
Total polyphenols	268.5 ± 15.4 (100)*	

Note: Values are means ± standard error of triplicate analysis.
*Relative abundance (% wt) on oleoresin DW basis.

Among the identified metabolites, some capsaicinoids and nerolidol were described to have potential antifungal property.

3.2 HPLC analysis of polyphenols in chili pepper oleoresin

The polyphenolic analysis of chili pepper oleoresin was obtained after a double extraction of pepper powder in 100 and 70% methanol, according to Jeong *et al.*[53] which allowed identification of 13 different polyphenols, such as phenolic acids components among cinnamic and benzoic derivatives, three quercetin derivatives as flavonols, and naringenin and its glucoside as flavanones, while flavone and flavanol components were not detected or present under limit of detection (Table 2). The most abundant compound on a DW basis was represented by vanillic acid (65%), followed by protocatechuic acid (13%), being hydroxycinnamic derivatives the most important fraction in terms of quantity (74%) in respect to the other polyphenol compounds. The total concentration of polyphenols in oleoresin accounted for 268.5 ± 15.4 μg mg^{-1} DW, quite similar to the amount reached by total fractions of capsaicinoids.

3.3 HPLC analysis of carotenoids and antioxidant potential of chili pepper oleoresin

The main carotenoid compounds present in chili pepper oleoresin were identified by comparing retention times (Rt) and UV–Vis spectra obtained by HPLC analysis with the corresponding parameters exhibited by the standards (Supporting Information, Fig. S2 and Table 3).

Oleoresin from the red and orange-yellow pigmented chili pepper varieties contained large abundance of both major yellow and red carotenoids, as *β*-carotene (0.59 μg mg^{-1} DW and 12.5% of relative peak area), capsanthin (0.22 μg mg^{-1} DW and 8.5%), zeaxanthin (0.06 μg mg^{-1} DW and 3.7%), yellow xanthophyll *β*-criptoxanthin (0.08 μg mg^{-1} DW and 3.1%), and epoxy-xanthophyll antheraxanthin (0.04 μg mg^{-1} DW and 2.9%) (Supporting Information, Fig. S2 and Table 3). Capsorubin, a carotenoid exclusively found only in red *Capsicum* species as well as the above mentioned capsanthin, and yellow epoxy-xanthophyll violaxanthin contributed only to a minor level, being under the Limit of Detection (LOD). A number of unidentified compounds were also detected at different retention times, contributing to almost 30% of the total peak area.

Table 3. HPLC analysis of principal carotenoids in chili pepper oleoresin

Compounds	Rt	Content in Oleoresin (μg mg^{-1} DW)	LOD (mg L^{-1})	Biological activity
Capsorubin	4.140	<LOD	0.01	
Violaxanthin	4.458	<LOD	0.06	Antioxidant[91]
Capsanthin	5.023	0.22 ± 0.02 (21.18)*	0.01	Antioxidant[91]
Antheraxanthin	5.472	0.04 ± 0.00 (4.05)*	0.01	Antioxidant[91]
Lutein	6.129	0.03 ± 0.00 (2.80)*	0.01	Antioxidant[91]
Zeaxanthin	6.508	0.06 ± 0.01 (6.32)*	0.01	Antioxidant[91]
IS	8.390	-		
β-Criptoxanthin	13.296	0.08 ± 0.01 (7.76)*	0.03	Antioxidant[91,92]
β-Carotene	30.077	0.59 ± 0.04 (57.44)*	0.02	Antimicrobial[33,91,93] Antioxidant[94]
Total Carotenoids		1.02 ± 0.07 (0.1)†		

Note: Retention time (Rt), relative content, Limit of Detection (LOD) and biological activity of the principal carotenoids detected in chili pepper oleoresin after HPLC analysis in acetonitrile: methanol (40:60). IS, internal standard.
*Relative abundance (% wt) on total identified carotenoids.
† Relative abundance (% wt) on oleoresin DW basis.

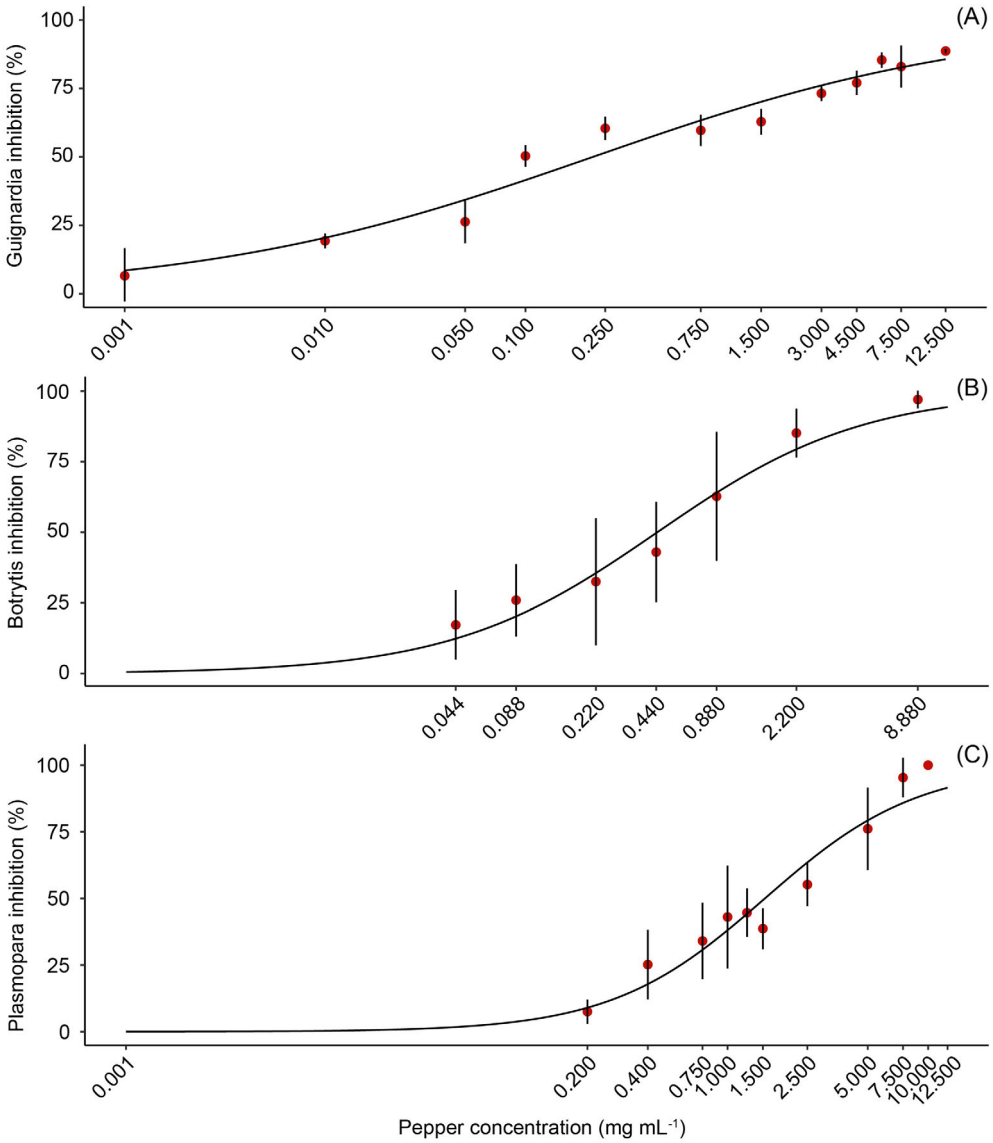

Figure 1. Antifungal activity of chili pepper oleoresin. Log-logistic dose–response curve illustrating the growth inhibitory effect of increasing pepper oleoresin concentrations on radial growth of *G. bidwellii* (A) and *B. cinerea* (B) after 6 and 2 days of incubation at 21 °C, respectively and on sporulation on grapevine leaf discs for *P. viticola* (C). Data are expressed as mean ± SD (n = 3). Lack-of-fit *P* value > 0.05.

By comparison with their relative internal standards, HPLC analysis showed that the total amount of the main carotenoids accounted for a very limited concentration (1.02 µg mg^{-1} DW) compared to capsaicinoids, contributing only to approximately 0.1% on oleoresin DW basis. This result was also confirmed by basic spectrophotometric method for carotenoid quantification on acetone-resuspended oleoresin, determining an almost similar amount of 0.692 ± 0.074 µg mg^{-1} DW (n = 3). The most represented pigments of the yellow and red fraction of carotenoids were *β*-Carotene and capsanthin (57 and 21% of the total carotenoids), respectively.

Finally, the overall reducing power of chili pepper oleoresin was assessed, accounting for the average value of 3704.48 ± 192.21 µmoles Trolox equivalents (TE) g^{-1} DW extract (ORAC).

3.4 Antifungal activity of chili pepper oleoresin on grapevine pathogens

After chemical characterization, antifungal activity of chili pepper extract at different concentrations was tested by *in vitro* assays against three grapevine pathogens (Fig. 1). Overall, the oleoresin exhibited significant inhibition activity against growth and sporulation of isolates from the hemibiotrophic ascomycete *G. bidwellii*, the necrotrophic *B. cinerea* and the biotrophic oomycete *P. viticola*, over a large range of concentrations, ranging from 0.001 to 12.5 mg mL^{-1}.

In particular, the inhibition of *G. bidwellii* mycelial growth on culture medium was measured at day 6, starting from sterilized 1 month-old plug, when it reached 89% at the highest dose of 12.5 mg mL^{-1} (Supporting Information, Fig. S3).

Further concentrations of the extract were not tested, due to difficulties in achieving homogenous dispersion of the oleoresin in water at higher concentrations and for the necessity to avoid excess of ethanol in the assay. With diluted different concentrations of oleoresin, it was possible to interpolate the curve shown in Fig. 1 panel A, and thus to calculate the concentration that inhibited 50% of *G. bidwellii* mycelium growth, *i.e.* ED_{50} of 0.233 ± 0.034 mg mL^{-1}.

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In the case of *B. cinerea*, inhibition of radial mycelium growth starting from sporulating conidia at day 2 was measured at several dilutions of extract (Supporting Information, Fig. S4).

In this case, chili pepper extract almost completely inhibited the pathogen at a lower dose of 8.8 mg mL^{-1}, however exhibiting a lower ED_{50} (0.445 ± 0.091 mg mL^{-1}) if compared to *G. bidwellii* (Fig. 1(B)). This difference could be related to differential sensitivities and/or detoxifying capacities possessed by the two fungal pathogens or their specific growth stages towards the bioactive compounds present in oleoresin extract.

Similarly, as for the previous pathogens, oleoresin was also effective in reducing the sporulation and evasion of the obligate biotrophic *P. viticola*, reaching 100% of inhibition at 12.5 mg mL^{-1} (Supporting Information, Fig. S5). Conversely, it seemed that its efficacy was less strong at lower doses, since higher value of ED_{50} of 1.535 ± 0.157 mg mL^{-1} was reported (Fig. 1(C)). It has to be noted that, even at the highest dosage, no apparent symptoms of phytotoxicity of pepper oleoresin on leaf discs were observed.

Overall, these findings could be very promising for the high efficacy shown by chili pepper oleoresin over a range of fungal diseases which pose increasing concern and challenges for grapevine pest sustainable management. Among the pathogens tested, the oleoresin could be particularly valuable for its potential bioactivity against *G. bidwellii*, since it showed the highest efficacy in terms of ED_{50} and it could be used in biocontrol of this new emerging disease.

4 DISCUSSION

4.1 Extraction of crude chili pepper oleoresin by eco-safe solvent

Reduction of fungicide dosage and use of conventional pesticides in vineyards is now recognized as a priority by government authorities, which encourage the development and adoption of sustainable alternatives. Besides agricultural practices and cultivation of resistant genotypes for limiting the risks of fungal pathogens spreading, the implementation of alternative treatments, including resistance inducers and biofungicides of both botanical[95,96] or bacterial origin,[97] is additionally required.

In this regard, chili peppers are rich in different polar bioactive compounds and several solvents could be used to extract them efficiently.[64,65] Therefore, a preliminary issue has been the best choice of solvent for oleoresin preparation. Accordingly, previous reports highlighted that solvent chemical properties such as polarity may determine variations on content of capsaicinoids, carotenoids and phenolic compounds,[65] and they influenced also chili pepper oleoresin antioxidant activity since the latter was strongly correlated with the content of bioactive compounds.[64] As mid-polar solvent, ethyl acetate has been still utilized in oleoresin extraction from hot and non-pungent peppers, attaining the highest levels of total capsaicinoids in comparison to other organic solvents, better than or following only to what resulted by extraction with hexane.[65,98] Lastly, considering that capsaicinoids are partially soluble in hexane and avoiding the use of highly toxic and inflammable solvents such as methanol and acetone, ethyl acetate was considered to be the best compromise for obtaining a 'green' hot chili pepper oleoresin extract with high levels of bioactive molecules. In accordance, ethyl acetate is scored as recommended in guidelines for solvent selection, given that it can be sourced renewably.[99]

4.2 Bioactive compounds of chili pepper oleoresin and antioxidant potential

From the results of this study, the oleoresin achieved from a mixture of hot pungent varieties of *C. chinense* fruits contained particularly high amounts of capsaicinoids and polyphenols (371 and 268 μg mg^{-1} DW, respectively, see Tables 1 and 2). In comparison, in fresh whole fruits from 18 different accessions of Habanero peppers the concentration of acetonitrile-extracted capsaicinoids ranged from 10 to 60 mg g^{-1},[100] similarly to the average content found in dry fruits.[101] Moreover, *C. chinense* has several times higher capsaicinoid content and hottest pungency compared to other *Capsicum* spp.,[102] being 'Carolina Reaper' the variety with the highest capsaicinoid content at 73.34 mg g^{-1} DW by methanolic extraction.[49]

Concerning polyphenols, *C. chinense* displayed on average 1350 mg 100 g^{-1} DW of total phenolic content by colorimetric method in whole dry fruits,[49] being even higher if placenta was considered with respect to other parts of the pericarp.[103] Interestingly, several authors stated a strong correlation between high polyphenols and capsaicinoids in *Capsicum* sp.,[49,104,105] which was mainly explained for that the phenylpropanoid and capsaicinoid biosynthetic pathways are converging during pepper fruit ripening.[104,106]

Carotenoids represent the third class of important bioactive metabolites we have investigated in this study, though in terms of abundance it remained a minor fraction, reaching only 1.02 μg mg^{-1} on DW basis (Table 3). Nonetheless, they contribute, together with capsaicinoids and polyphenols, to the total antioxidant potential of chili pepper oleoresins.[105,107] Wahyuni *et al.*[24,108] reported an average total carotenoid content of approximately 40 mg 100 g^{-1} fresh weight (FW) in different *Capsicum* accessions, whereas others found large variation for carotenoid concentration and composition.[109,110] In dry fruit, carotenoids content ranged from 0.1 to 3.2 g 100 g^{-1}.[28] Chili peppers, as 'Habanero' and 'Naga Morich', were also particularly rich in β-carotene,[107] which explained the major contribution of this yellow pigment on the oleoresin quantified in the present study, followed by capsanthin, characteristic of *Capsicum* sp., which generally contributes to a large fraction of carotenoids in most of the varieties.[28]

In this study, all the previous major phytochemicals mentioned may contribute to the total antioxidant activity of the oleoresin with a mean ORAC value of 370 448 ± 19 221 μmoles TE 100 g^{-1} DW, which is very similar to or even higher than the values reported for cayenne pepper[111,112] and quite lower than *C. frutescens* extracts.[113] A reliable comparison on literature reports is however very difficult to be achieved, due to high variation in the ORAC values in reliance on *Capsicum* variety, origin, fruit processing and solvent used for product extraction. Moreover, it has to be underlined that the oleoresin used in the present study was originating from an assortment of seven different varieties of *C. chinense*, whose particular contribution to the total antioxidant value of the oleoresin is difficult to disentangle. Nonetheless, it is proposed that a significant component of the antioxidant activity in the chili pepper oleoresin used in this study could be attributed mainly to the large abundance of capsaicinoids and polyphenols, given that this activity in *Capsicum* has been positively related to the latter compounds.[65,103,104,114,115]

4.3 Antimicrobial activity of chili pepper oleoresin

Apart from the high antioxidant value of the oleoresin, the results of *in vitro* pathogen growth assays evidenced that the extract is

effective, at different levels, on mycelial growth or sporulation of three important pathogens of grapevine (Fig. 1). In particular, comparison of dose–response curve patterns and ED_{50} of pathogens considered highlights that the highest doses of oleoresin (in the range of 4.5–12.5 mg mL^{-1}) did not cause a 100% inhibition of mycelial growth in the case of black rot agent, *G. bidwellii*, differently from what observed for *P. viticola* and *B. cinerea*. This feature might suggest that oleoresin exerted more a fungistatic than a fungitoxic activity on this Ascomycete. Hence, using a pharmacology definition, the oleoresin exhibited a higher 'efficacy' in inhibiting *P. viticola* and *B. cinerea* mycelial growth (potential fungitoxic action) at higher concentrations, but a stronger 'potency' against *G. bidwellii* since the ED_{50} is reached at a lower dose.[116] However, this fungus appeared to be particularly sensitive at lesser concentrations of chili pepper extract, being its radial mycelial growth decreased by 25% even at a low dose of 0.05 mg mL^{-1}. This observation was confirmed by the lowest calculated value of ED_{50} (0.233 mg mL^{-1}), which doubled in *B. cinerea*, followed by the far higher level assessed for *P. viticola* sporulation.

To the best of our knowledge, this is the first report of fungicidal effect by *Capsicum*-based extract on the black rot of grapevine, since there is no available information from literature about specific fungicidal efficacy by the specific capsaicinoids or phenolic compounds we have identified so far in the chili pepper oleoresin. In fact, in searching of natural alternatives for organic wine-production, black rot agent was only shown to be sensitive to saponin-containing botanicals on *in vitro* experiments, where a high level of inhibition of conidial germination was provided by *Primula* and *Hedera helix* root extract and other species at concentrations below 0.5% (w/v aqueous or ethanolic solution).[13] Moreover, both protective and curative efficacy of the extract was also demonstrated by greenhouse tests on potted vines under controlled conditions in the same study. The bioactive compounds mainly responsible for inhibition of conidia germination are ascribed to saponins, which unfortunately exhibited poor efficacy under field conditions, due to the high water-solubility of their formulation. Detergent properties of saponins are believed to explain the possible lytic and fungicidal action on fungal spores.[117] Saponin identification in the pepper oleoresin was out of the scope of the present study, however, interestingly, Lucca *et al.*[118] isolated a potent fungicidal saponin from dry ground *Capsicum* sp. fruit, effective against *Aspergillus* sp. germinating conidia.

Therefore, without any additional information, we could only suppose that the most abundant compounds detected in oleoresin, *i.e.*, capsaicinoids (capsaicin and dihydrocapsaicin) and hydroxycinnamic acids (mainly vanillic) and protocatechuic acid among polyphenols, might be responsible for fungicidal action on *G. bidwellii*, although we cannot exclude *a priori* that also other minor bioactive components could contribute in a concerted manner with the above mentioned phytochemicals.[85]

Regarding the efficacy of the oleoresin on conidia sporulation and radial mycelial growth of the necrotrophic agent *B. cinerea*, the pathogen was found to be susceptible to increasing oleoresin doses, showing a quite low value of ED_{50} and displaying maximal activity at just the concentration of 8.8 mg mL^{-1}. Similarly, also *P. viticola* sporulation was inhibited completely by pepper oleoresin, without any evident phyto-toxicity, though at higher concentrations it showed quite high ED_{50} value. In accordance, some authors reported that *B. cinerea* and some oomycetes species were highly susceptible to capsacinoids,[39,41,43] suggesting that the lateral chain of capsaicinoids compared to the phenolic one could explain high fungal inhibition, due to osmotic stress and phospholipid membrane damage.[43,119] Moreover, several data from literature are consistent with the efficacy of phenolic acids, and specifically ferulic and vanillic acid, in inhibiting filamentous fungi.[86] Protocatechuic acid, from both bacteria or plant origin, displayed strong inhibition against some major postharvest pathogenic fungi as well as on *B. cinerea* colonization of strawberry fruits, probably by causing fungus membrane permeability impairment.[87]

Interestingly, GC–MS analysis of chili pepper oleoresin run in dichloromethane has identified other minor bioactive components, known from literature to exert some antimicrobial and antifungal activities apart from capsaicinoids, such as essential oils (Table 1). In particular, among volatile compounds, nerolidol displayed remarkable antifungal action against most important phytopathogens (*Fusarium*, *Phytophthora*, *Colletotrichum*, *Alternaria*, *Rhizopus*, *Penicillium*, *Sclerotium* and *Rhizoctonia* species).[76,77,120] This volatile compound (3,7,11-trimethyl-1,6,10-dodecatrien-3-ol) is a sesquiterpene naturally present in many plant species, with large application in food and cosmetic industries.[121] In this regard, the single compound and its derivatives displayed bactericidal and antifungal properties: in particular, *trans*,*trans*-farnesol against *Phomopsis obscura*[78] and nerolidol are effective against *in vitro Sclerotium cepivorum* growth, ranging from 2.0 to 5.0 μg per disc.[77,79] Although very raw, a comparison among these data and our results allows evidence that at the highest dosage of 12.5 mg mL^{-1} of oleoresin used against *G. bidwellii*, the ascomycete encountered approximately 1.68 μg per fungal disc, giving a very plausible contribution to the oleoresin antifungal efficacy. They reduced sclerotia formation and caused strong fungal growth inhibition by alterations of mycelial morphology and membrane permeability, possibly through their insertion into lipid membranes due to their high hydrophobicity. Consistently, a number of published studies have demonstrated that terpene synthase activity increased and monoterpene and sesquiterpene accumulation took place in response to pathogen's attack[80,122] and recently (*E*)-nerolidol has been reported to act also as a volatile signal enhancing the natural plant's immune system in tea plant.[81] Taken together, all of this evidence might suggest that also some other bioactive compounds, although represented in minor quantity in the oleoresin, may be related to the high antifungal potential exerted against the three pathogens of grapevine. Furthermore, the co-presence of several bioactive compounds with high antioxidative properties in the complex mixture of the oleoresin might even represent an advantage if compared to the use of a single, isolated active compound. For example, the industrial utilization of capsaicin has been limited by its low water solubility shown by one-single formulation and by its degradation under extreme environmental conditions, thus requiring its incorporation into specific matrices or nanoformulates.[123,124]

5 CONCLUSION

These results, though preliminary, demonstrated that the oleoresin from chili peppers is able to inhibit the growth of some of the most harmful pathogens in grapevine. At least, capsaicinoids and phenolic acids found at high content, associated to other biologically active components are all likely responsible for the activity against all three phytopathogens investigated.

Noteworthy, the high potential shown by the pepper oleoresin to specifically contrast *G. bidwellii*, the causal agent of black rot of grapevine, may deserve more investigations to produce it as new alternative or complementary bio-fungicide. The diffusion

of the latter disease has recently emerged as one of the major concerns in modern viticulture, due to the combination of restricted limitations in extensive use of copper by EU and introduction of mildew-resistant hybrids in organic vineyard, making it more vulnerable to infections from secondary fungal pathogens and ongoing yield losses. Nonetheless, it is still required to gain more information about the mechanism of action of this extract and to further explore the suitability of this material in vineyard, as well as to improve and achieve the best formulation for its application to vine plant.

Regarding pepper oleoresin manufacturing for pest management, it has to be said that the increase in the total dry pepper production by 30% during the last decade (2011–2021) (FAOSTAT 2022) is an attempted response to the enhanced request for its wide industrial applications in pharmaceutical and cosmetical sectors. Hence, the implementation in industrial production of habanero peppers, together with the development of new breeding programs of F1 hybrids very rich in bioactive compounds,[125] could really make the oleoresin production more sustainable, answering to the required necessity of new eco-friendly products for pest management. Other technological approaches aiming at increasing the sustainability of the pepper oleoresin application would be the exploration for the optimized industrial size process such as carbon dioxide supercritical extraction systems[126] or the use of chili pepper by-products such as discarded vegetative parts, peduncles[89] or fruit waste generated by seed production (coat debris, small seeds.) as source material, in the framework of a circular economy strategy.

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CONFLICT OF INTEREST STATEMENT

All the authors confirmed that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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