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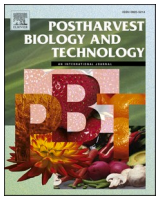

Volatile organic compounds released by chitosan formulations present diverse chemical composition and produce differential effects on postharvest pathogens

Samuel Álvarez-García [a,b], Ilaria D'Isita [c], Onofrio Marco Pistillo [c], Marwa Moumni [a], Giacinto Salvatore Germinara [c], Gianfranco Romanazzi [a,*]

[a] Department of Agricultural, Food and Environmental Sciences, Marche Polytechnic University, Ancona, Italy
[b] Plant Physiology Area, Engineering and Agricultural Sciences Department, Universidad de León, León, Spain
[c] Department of Agricultural Sciences, Food, Natural Resources and Engineering (DAFNE), University of Foggia, Foggia, Italy

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ABSTRACT

Chitosan is a natural biopolymer used as a sustainable alternative to synthetic pesticides in agriculture due to its antimicrobial activity against postharvest pathogens, its ability to enhance plant defensive responses, and the formation of a protective film on the plant surface. Little is known regarding the nature of volatile organic compounds (VOCs) in chitosan formulations and their antimicrobial potential. In this study, we evaluated the antifungal activity of VOCs from nine chitosan formulations against *Botrytis cinerea*, *Monilinia fructicola*, *Monilinia laxa*, *Alternaria alternata*, and *Alternaria brassicicola*. The VOC composition of the tested formulations was analyzed by gas chromatography coupled with mass spectrometry (GC-MS), and the relative abundance of the different compounds was correlated with the antifungal activity using a hierarchical cluster analysis (HCA). VOCs released by chitosan formulations presented diverse chemical composition and produced differential growth inhibitory effects on the tested fungi. This activity seems to be dose dependent, although not directly linked to chitosan concentration, its chemical nature, or source. HCAs indicated a strong correlation between dichloromethane and, especially, phenol, 2,5-di-tert-butyl with the antifungal activity observed against all five tested postharvest pathogens, while *M. laxa* inhibition correlated also with 3-hexanone, 2-hexanone, and 2-pentanone. This study opens a new road for further research and characterization of VOCs from chitosan formulations, their biological activities, and their potential use in postharvest disease management.

1. Introduction

Food crops worldwide face losses of up to 40 % every year due to pests and plant diseases, according to the latest estimates of the Food and Agriculture Organization of the United Nations. Crop losses caused by plant disease alone cost the global economy € 200 billion annually (Food and Agriculture Organization of the United Nations FAO. 2021). Pre and postharvest diseases caused by fungi such as *Botrytis cinerea*, *Monilinia* spp., *Alternaria alternata*, and *Alternaria brassicicola* contribute to the loss of fruit and vegetables. *Botrytis cinerea*, causal agent of gray mold, is a particularly destructive pathogen affecting a wide range of crops (Williamson et al., 2007; Romanazzi et al., 2016). *Monilinia* species cause brown rot, mainly *Monilinia laxa*, *Monilinia fructigena*, and *Monilinia fructicola*, and this disease is of economic importance in major stone fruit producing areas worldwide (De Miccolis Angelini et al., 2022). *Alternaria alternata* and *A. brassicicola* are seedborne and postharvest pathogens primarily affecting *Cucurbita* spp. and *Brassica* spp., respectively (Palou and Smilanick, 2019; Moumni et al., 2020; Zhang et al., 2022).

The control of these diseases is becoming ever more challenging as environmental factors put pressure on agricultural systems and the traditional use of chemical synthetic pesticides is limited or banned due to diverse ecological, technical, health, social, legal, and economic considerations (Sivakumar et al., 2021). As a result, the search for new and more natural alternative methods for plant disease control is gaining traction, including the use of non-volatile and volatile organic compounds (VOCs) with antimicrobial activity (Ribes et al., 2018; El Khetabi et al., 2022; Razo-Belman and Ozuna, 2023; Liang et al., 2025). VOCs

* Corresponding author.
E-mail address: g.romanazzi@univpm.it (G. Romanazzi).

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are small natural molecules with specific characteristics of molecular weight (MW) and vapor pressure that make them appear in gaseous state at environmental conditions. They are synthetized and released by a variety of organisms, and mediate or influence several intra- and inter-specific interactions (Tilocca et al., 2020; Duc et al., 2022). One of the main focuses of VOCs application regards their use in plant protection and the biological control of crop diseases and pests (Herrera et al., 2015; Tilocca et al., 2020; Derbassi et al., 2022; Razo-Belman and Ozuna, 2023). VOCs have proven to exert strong antimicrobial activity against many economically relevant fungal postharvest pathogens, including *B. cinerea*, *Rhizoctonia solani*, *Penicilium* spp., *Alternaria* spp., *Monilinia* spp., *Rhizopus* spp., *Aspergillus* spp., *Fusarium* spp., among others (Arrarte et al., 2017; Božik et al., 2017; Yang et al., 2018; Di Francesco et al., 2020; Li et al., 2022; Álvarez-García et al., 2023). This antimicrobial activity has led many researchers to propose the use of VOCs as part of integrated disease and pest management strategies, especially in postharvest stages (Derbassi et al., 2022).

Chitosan is a natural biopolymer resulting from the deacetylation of chitin. Structurally, it is a linear polysaccharide formed by N-acetyl-glucosamine and β-1,4-D-glucosamine units linked via β-1,4-glycosidic bonds (Kean and Thanou, 2011). The fraction of units presenting free amino groups determines its degree of deacetylation, which has been demonstrated to be a key factor in its bioactivity. Most commercial chitosan formulations present a degree of deacetylation ranging between 70 % and 90 % (Kou et al., 2022). Another relevant characteristic that defines chitosan properties and bioactivity is MW. Water solubility and biological activity of chitosan increases as its MW decreases (Pillai et al., 2009; Younes and Rinaudo, 2015). Chitosan can be obtained from a range of eukaryotic organisms such as fungi, insects, mollusks, and crustaceans.

Due to its biocompatibility, biosafety, and bioactivity, the use of chitosan has reached many fields, including cosmetics, medicine, pharmacology, biotechnology, food processing, and agriculture. Chitosan hydrochloride was one of the first basic substances approved in 2014 by the European Union (Reg. EU, 2014/563), and a second chitosan formulation was approved in 2022 (Reg. EU, 2022/456). Basic substances are compounds that can be used in plant protection as sustainable alternatives to synthetic pesticides (Romanazzi et al., 2022). They do not pose health or environmental concerns, do not have maximum residue limit (MRL) and, mostly, no preharvest interval (Charon et al., 2019). Basic substances can complement or replace the use of synthetic pesticides with benefits for the users, the environment, and the consumers (Marchand et al., 2021; Romanazzi et al., 2024).

Chitosan and chitosan-based formulations presents a triple action in plant protection; namely its ability to activate or enhance plant defensive responses, exert antimicrobial activity against a wide range of plant pathogens, and form a protective film on the treated plant surface (Romanazzi et al., 2019). Chitosan has shown a strong capacity to control pre and postharvest decay and foodborne diseases (Heinzen et al., 2022; Toffolatti et al., 2023), extend shelf life of agricultural products, and overall reduce fruits and vegetables waste (Romanazzi and Moumni, 2022). Chitosan can be applied in postharvest stages in the form of edible coatings, micellar systems, and nanoparticles, both alone or as a carrier for the delivery of other substances, including non-volatile and volatile organic compounds (VOCs) with antimicrobial activity (Heinzen et al., 2022; Heras-Mozos et al., 2022a,b; Morales-Rabanales et al., 2022).

In this context, although strong attention has been placed on chitosan structure, formulation, application, and bioactivity; very little is known regarding the VOCs released by chitosan formulations themselves and their potential biological activities.

Being a biopolymer with high MW, at first glance pure chitosan does not seem to be a prime candidate for VOC emission in environmental conditions. Since chitosan formulations are the result of processing, extraction, chemical modification, and successive formulation of natural products, they may contain volatile molecules remaining from the original natural source or derived from the subsequent transformation steps. These VOCs could potentially present diverse biological activities, and thus we propose that they should be considered when fully characterizing chitosan-based products and their effects.

In the present study, we hypothesize that commercial chitosan-based products release VOCs with biological activity, and more specifically with antimicrobial effects. Therefore, our main goals were: i) to evaluate *in vitro* the antimicrobial activity of VOCs released by chitosan-based commercial formulations against five fungal phytopathogens (*B. cinerea*, *M. fructicola*, *M. laxa*, *A. alternata*, and *A. brassicicola*); ii) to identify their VOC composition using static headspace solid-phase micro-extraction (HS-SPME) followed by gas chromatography coupled with mass spectrometry (GC-MS); and iii) to correlate the chemical composition of the chitosan formulations with their antimicrobial activity *in vitro*.

2. Material and methods

2.1. Fungal strains and chitosan formulations

Five phytopathogenic fungi were evaluated in the present study. Namely, *B. cinerea* (strain B05.10; Netherlands; GenBank accession: PRJNA15632), *M. fructicola* and *M. laxa* (both isolated from infected nectarines in Italy and previously identified in our laboratory using multiplex PCR (Makau et al., 2023)), *A. alternata* IA3 (isolated from *Cucurbita moscata* seeds; GenBank accession: MK497776), and *A. brassicicola* (isolated from *Brassica oleracea* seeds; Collection of Plant Pathology Unit, Marche Polytechnic University, Italy). They were stored at 4 ºC and refreshed in a growth chamber (Lovibond TC255S, Tintometer GmbH, Germany) at 22 ºC ± 2 ºC on potato dextrose agar (PDA; 40 g/L; Scharlab S.L., Sentmenat, Spain) before being used in the experiments. Nine commercial chitosan formulations were tested against them in different concentrations (Table 1).

2.2. Antifungal activity of VOCs released by chitosan formulations

The five fungal strains were exposed to the VOCs released by the chitosan formulations at a concentration of 1 %, 5 % (w/v) (except for those with a lower commercial concentration), and at their original commercial concentration (apart from CH5 and CH8, that were diluted to 10 % w/v) (Table 1). The resulting chitosan concentrations were obtained by diluting the commercial formulation in the corresponding volume of distilled water.

Non-vented VOC Chambers (Álvarez-García et al., 2021) (kindly provided by the GUIIAS Group, Universidad de León, Spain) were used to carry out the experiments in the following way (Fig. 1): 10 mL of the corresponding chitosan formulation were placed into 90 mm Petri dishes, then the lids were removed and substituted with a perforated VOC chamber central piece. Fungal plugs with a diameter of 8 mm were inoculated in the center of Petri dishes containing 20 mL of PDA, their lids were removed, and the dishes were placed upside-down over those containing the chitosan formulation, thus forming the full closed chambers (Fig. 1 and Fig. 2). They were subsequently sealed with two layers of Parafilm (Amcor-Bemis, USA) and incubated at 20 ± 2 ºC. Inoculated plates exposed to 10 mL of sterile water were used as control. Mycelial growth was recorded measuring two perpendicular colony diameters per replicate. These data were collected immediately before the colonies in the fastest growing treatment reached the edge of the plate, which corresponded to 3 days post inoculation (dpi) for *B. cinerea*, 5 dpi for *M. fructicola* and *M. laxa*, and 7 dpi for *A. alternata* and *A. brassicicola*.

Percentages of mycelial growth inhibition (PI) was calculated with the following equation: PI = [(C - T) / C] x 100, where C is the colony diameter in the control and T the diameter in the treatments, both after subtracting the diameter of the inoculation plug (8 mm). Five replicates were performed for the control and the 1 % concentration treatments, and three replicates for the higher concentrations. The experiments were

Table 1
Information regarding the chitosan formulations tested in the present study including their assigned codes.

Code	Active principle	Commercial concentration % (w/v)	Source	Product	Company	Tested concentrations
CH1	Chitosan hydrochloride	5 %	crustaceans & mollusks	Prevatect	Ascenza Agro S.A, Italy	1 %, 5 %
CH2	*COS-OGA	1.25 %	fungi-plants	Ibisco	Gowan Srl, Faenza, Italy	1 %, 1.25 %
CH3	Chitosan	1.9 %	crustaceans	Biorend	Bioplanet Srl, Cesena, Italy	1 %, 1.90 %
CH4	Chitosan hydrochloride	5 %	crustaceans & mollusks	Serbios	Serbios Srl, Badia Polesine, Italy	1 %, 5 %
CH5	Chitosan hydrochloride	50 %	crustaceans	Chitosano denso	Agrilaete Srl, Palmanova, Italy	1 %, 5 %
CH6	Chitosan hydrochloride	11.5 %	crustaceans	Kitae	GreenIMPULSE	1 %, 5 %, 11.5 %
CH7	Chitosan	10 %	*Aspergillus niger*	KITOGREEN DIRECT F1	Kitozyme SA	1 %, 5 %, 10 %
CH8	Chitosan	85 %	*Aspergillus niger*	KITOGREEN MASTER	Kitozyme SA	1 %, 5 %, 10 %
CH9	Chitosan	10 %	*Aspergillus niger*	KITOGREEN DIRECT F2	Diachem S.p.A.	1 %, 5 %, 10 %

*COS (Chito-oligosaccharides) - OGA (oligo-galaturonides).

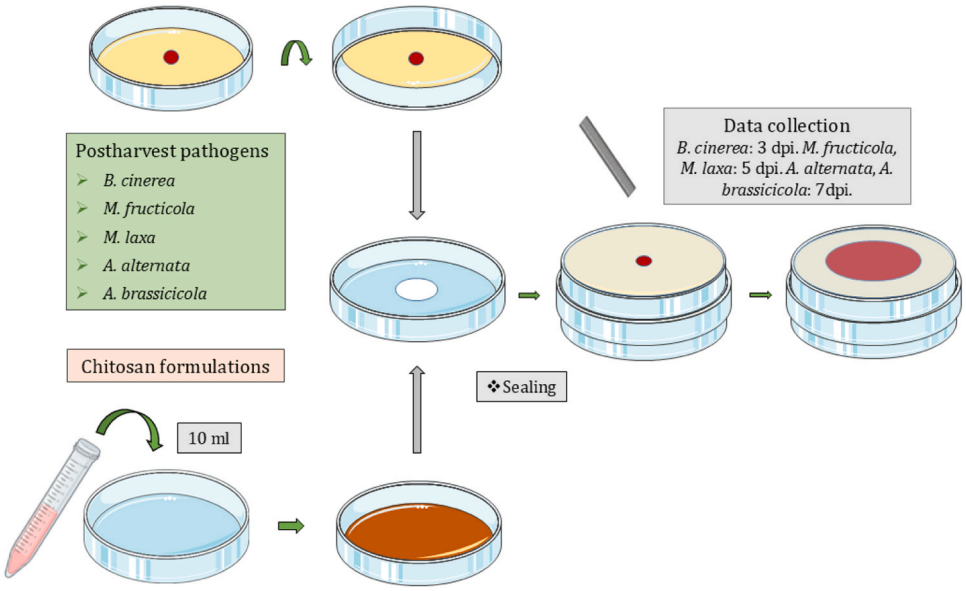

Fig. 1. Graphical representation of the protocol using non-vented VOC chambers (Álvarez-García et al., 2021) to evaluate the antifungal activity of VOCs released by the nine chitosan formulations on the tested fungal pathogens. Dpi: days post inoculation.

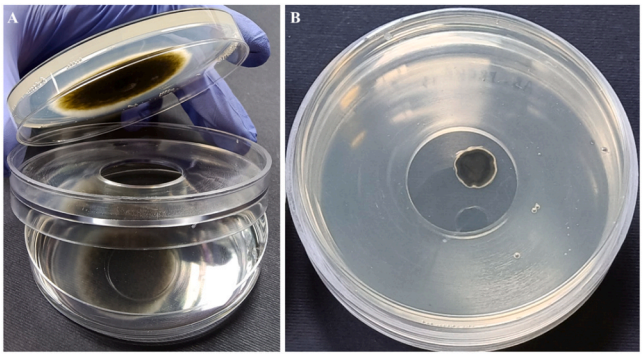

Fig. 2. VOC chamber with 10 mL of chitosan formulation in the lower Petri dish and a fungal pathogen growing on 20 mL of PDA in the upper plate. The hole in the central piece allows the free flow of VOCs between plates. A) Open chamber. B) Closed chamber.

carried out twice in the same conditions.

The fungal plugs from treatments presenting 100 % inhibition at the end of the assay were reinoculated on fresh PDA to determine the potential fungicidal or fungistatic activity of the chitosan VOCs. Eventual growth was recorded 7 dpi. After this period, if the fungus showed any mycelial growth the treatment was considered fungistatic, whereas if there was no growth at all it was considered fungicidal (Moumni et al., 2021).

2.3. VOCs analysis

Volatile compounds of chitosan samples at a concentration (w/v) of 1 % were determined by static headspace solid-phase micro-extraction (HS-SPME) technique, similar to that used in previous studies (Alves et al., 2020; Testa et al., 2022). Briefly, 2.5 mL of each sample was placed into a 4 mL headspace vial (Supelco Co., Bellefonte, PA, USA), sealed with a PTFE/silicon septum (Supelco Co., Bellefonte, PA, USA). Vials were heated at 35°C for 4 h. in a water bath prior to SPME headspace sampling. Extractions were performed using a SPME fiber (Supelco Co., Bellefonte, PA, USA) coated with either 50/30 μm of divinylbenzene– carboxen–polydimethylsiloxane (DVB–CAR–PDMS).

The fiber was conditioned before use by heating it in the injection port of the GC system at 270°C for 1 h. After the extraction time, the fiber was recovered and transferred to the injection port of the GC, where the compounds were thermally desorbed at 250°C for 4 min. A fiber cleaning step of 10 min at the conditioning temperature with the split valve opened was performed in the GC injector after every chromatographic run to remove any absorbed residue. Before the acquisitions, a blank test was performed under the same experimental conditions to check for possible impurities. GC–MS analyses were performed using an Agilent 7890B series gas chromatograph (Agilent Technologies, Milan, Italy) coupled with an Agilent 5977 A mass selective detector (MSD) equipped with an HP-5MS capillary column (30 m × 0.25 mm ID, 0.5 μm film thickness, J&W Scientific Inc., Folsom, CA, USA). The desorption step was carried out in the splitless mode (5 min) with a programmed temperature from 60°C to 250 °C at 5 °C/min, with a final holding time of 15 min. The carrier gas was helium at a flow rate of 1.25 mL/min. Spectra were recorded in the electron impact mode (ionization energy, 70 eV) in a range of 15–550 amu at 2.9 scans/s. The identification of volatile compounds was achieved by comparing mass spectra with those of the data system library (NIST11, p > 90 %) and, wherever possible, by comparing retention times (R.T.) and mass spectra with those of commercially available standards. A mixture of a continuous series of straight-chain hydrocarbons, C5–C40 (Alkane Standard Solution C5–C40, Sigma Aldrich, Milan, Italy), was injected into an HP-5MS column under the same conditions previously described for the chitosan samples to obtain the Linear Retention Indices (RIs) (Van Den Dool and Kratz, 1963). The relative abundance of each compound was calculated using the integrated peak area data from the GC-MS trace. Each extract was analyzed in triplicate.

2.4. Data treatment and statistical analyses

The Hierarchical Cluster Analysis (HCA), an unsupervised method that group different samples based on their similarity and does not require prior knowledge on the number of clusters to be formed (Tufariello et al., 2022), was run with the aim of grouping the chitosan formulations in dependence of mean percentages of VOCs GC peak areas and percentages of mycelial growth inhibition recorded for the tested phytopathogenic fungi.

Statistical analyses were performed using one-way analysis of variance (ANOVA), and means were separated with Tukey's post hoc test ($p \leq 0.05$). When data were not homoscedastic and/or normally distributed, non-parametric Kruskal–Wallis H-test was used followed by Mann-Whitney U-test ($p \leq 0.05$). All statistical analyses were performed using IBM SPSS Statistics 26 (Armonk, NY, United States).

3. Results

3.1. Antifungal activity of VOCs released by chitosan formulations

Among the nine chitosan formulations tested in this study, only VOCs from CH1, CH7, and CH9 presented a consistent general growth inhibitory activity against all fungal strains (Table 2, Fig. 3). CH1 at 5 % completely inhibited *M. fucticola, M. laxa*, and *A. alternata* growth; and produced around 97 % inhibition on *B. cinerea* and *A. brassicicola*. The same formulation at 1 % concentration also significantly reduced mycelial growth of all tested strains in comparison to the untreated control, with a PI of 64.5 % on *B. cinerea*; 48.4 % on *M. fructicola*; 29.4 % on *M. laxa*; 48.9 % on *A. alternata*, and 35.4 % on *A. brassicicola*. CH7 at 10 % concentration produced a PI of 66.1 % on *B. cinerea*; 58.2 % on *M. fructicola*; 70.9 % on *M. laxa*; 69.0 % on *A. alternata*, and 99.7 % on *A. brassicicola*, whereas the same product at 5 % produced PIs between 41.1 % and 9.9 %. Meanwhile, CH9 at 10 % concentration produced a PI of 65.9 % on *B. cinerea*; 100 % on *M. fructicola*; 100 % on *M. laxa*; 62.5 % on *A. alternata*, and 97.8 % on *A. brassicicola*. These last two formulations did not demonstrate significant antifungal activity at 1 % concentration but for CH9 against *M. laxa*, with a PI of 21.9 %. CH3 at commercial concentration (1.9 % w/v) and at 1 % significantly inhibited *B. cinerea* growth, with a PI of 24.0 % and 13.2 %, respectively. CH2, CH4, and CH5 produced a small but significant growth inhibition on *B. cinerea*, with PI of 20.5 %, 16.2 % and 14.4 %, respectively. Finally, all three concentrations of CH8 slightly but significantly inhibited the

Table 2
Mycelial growth (mm) of the five fungal strains exposed to the VOCs from the tested chitosan formulations on the mycelial growth of *Botrytis cinerea*, *Monilinia fructicola*, *Monilinia laxa*, *Alternaria alternaria*, and *Alternaria brassicicola*.

	Mycelial growth (mm)				
Treatment	*B. cinerea*	*M. fructicola*	*M. laxa*	*A. alternata*	*A. brassicicola*
Control	71.9 ± 1.2 ab	65.3 ± 1.2 def	70.6 ± 1.0 abcd	53.8 ± 2.8 abcd	53.4 ± 1.0 bcd
CH1 com[1]	2.2 ± 2.6 h	0.0 i	0.0 l	0.0 h	1.4 ± 1.8 h
CH1 1 %	25.5 ± 4.9 g	33.7 ± 4.9 g	49.8 ± 2.8 hi	27.5 ± 2.3 fg	34.5 ± 1.4 g
CH2 com	57.2 ± 2.6 de	74.7 ± 2.8 ab	75.8 ± 1.3 a	54.1 ± 5.1 abcd	55.8 ± 3.3 bc
CH2 1 %	64.8 ± 5.0 bcd	66.9 ± 1.9 cde	67.9 ± 3.5 cd	53.5 ± 6.4 abcd	49.5 ± 4.4 cd
CH3 com	54.7 ± 7.5 e	76.2 ± 3.7 a	74.3 ± 3.5 ab	50.3 ± 6.6 abcde	56.6 ± 4.5 b
CH3 1 %	62.0 ± 5.5 cde	63.6 ± 2.6 def	65.1 ± 2.1def	48.1 ± 5.0 abcde	48.0 ± 3.9 de
CH4 com	60.3 ± 3.5 cde	73.4 ± 1.7 abc	76.0 ± 1.0 a	51.9 ± 4.5 abcd	57.1 ± 1.5 b
CH4 1 %	67.4 ± 4.9 abc	62.1 ± 3.5 def	66.3 ± 2.5 d	45.1 ± 7.8 bcde	48.1 ± 4.0 d
CH5 10 %	61.5 ± 3.8 cde	74.8 ± 2.9 ab	73.6 ± 3.5 ab	52.6 ± 7.4 abcd	55.3 ± 1.3 bc
CH5 1 %	65.1 ± 6.9 bcd	65.9 ± 3.0 cdef	68.8 ± 3.4 bcd	44.5 ± 4.9 cde	50.0 ± 3.0 cd
CH6 com	75.2 ± 1.6 a	64.5 ± 3.5 def	74.0 ± 2.5 ab	46.0 ± 4.9 bcde	52.2 ± 0.8 bcd
CH6 5 %	76.0 ± 1.0 a	63.7 ± 2.3 def	73.3 ± 1.0 abc	53.7 ± 3.8 abcd	52.7 ± 0.8 bcd
CH6 1 %	76.6 ± 0.9 a	64.5 ± 0.8 def	73.6 ± 0.7 ab	56.5 ± 3.3 ab	52.4 ± 1.4 bcd
CH7 com	24.3 ± 1.9 g	27.3 ± 2.0 g	20.5 ± 1.0 k	16.7 ± 0.8 g	0.2 ± 0.3 h
CH7 5 %	42.3 ± 3.5 f	58.8 ± 6.0 f	49.0 ± 2.6 i	39.0 ± 1.8 ef	37.2 ± 1.0 fg
CH7 1 %	67.4 ± 1.2 abc	68.7 ± 1.8 abcd	65.4 ± 1.6 de	54.3 ± 4.3 abcd	57.7 ± 2.7 ab
CH8 10 %	77.0 ± 0.0 a	66.8 ± 1.5 cde	57.3 ± 0.3 g	58.8 ± 0.3 a	57.0 ± 1.8 b
CH8 5 %	75.3 ± 0.3 a	67.8 ± 1.5 bcd	59.5 ± 0.5 fg	59.3 ± 1.6 a	53.8 ± 0.3 bcd
CH8 1 %	73.3 ± 0.7 ab	66.7 ± 2.4 cde	60.4 ± 0.8 efg	55.5 ± 4.0 abc	54.0 ± 2.8 bcd
CH9 com	24.5 ± 1.3 g	0.0 i	0.0 l	20.2 ± 2.9 g	1.2 ± 0.6 h
CH9 5 %	41.3 ± 2.0 f	15.2 ± 11.9 h	27.0 ± 1.3 j	43.0 ± 3.5 de	41.5 ± 1.3 ef
CH9 1 %	68.9 ± 2.0 abc	59.9 ± 1.2 ef	55.1 ± 2.9 gh	52.8 ± 4.4 abcd	64.0 ± 2.1 a

The results represent mycelial growth and are expressed as the mean ± standard deviation. Statistical analyses were carried out using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test ($p \leq 0.05$) or non-parametric Kruskal–Wallis H-test, followed by Mann-Whitney U-test ($p \leq 0.05$). Different lower-case letters indicate statistical differences between treatments on the same fungal strain.
[1] commercial concentration for each chitosan formulation (see Table 1 for details).

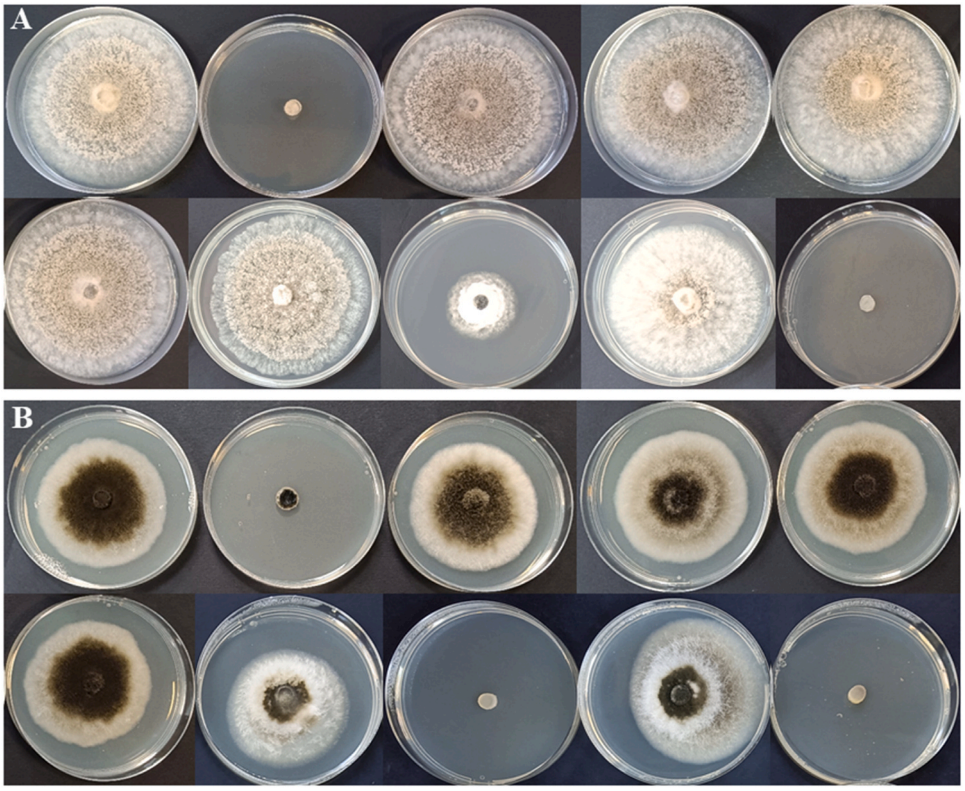

Fig. 3. Representative examples of A) *Monilinia fructicola*, and B) *Alternaria brassicicola* exposed to VOCs from chitosan formulations at their highest tested concentration (see Table 1 for details). Order of treatments from top left to bottom right: Control, CH1, CH2, CH3, CH4, CH5, CH6, CH7, CH8, CH9.

growth of *M. laxa*, with PIs ranging from 14.4 % to 18.8 %. None of the other tested chitosan formulations and concentrations produced statistically significant growth inhibition on any of the fungi. *M. fructicola* colonies exposed to VOCs from CH2, CH3, CH4, and CH5 at their highest concentrations showed statistically higher growth diameters than the untreated control. The same effect was verified for CH9 at 1 % concentration on *A. brassicicola* (Table 2).

CH1 at commercial concentration presented fungicidal activity against *M. fructicola*, *M. laxa*, and *A. alternata*, since the fungal plugs previously exposed to VOCs from this treatment did not grow when re-inoculated on fresh PDA. Effects of CH1 on *B. cinerea* and *A. brassicicola* and the other compounds on all five fungi were fungistatic.

3.2. VOCs analysis

A total of 28 VOCs were identified in the headspace of the studied chitosan formulations, classified in 11 aldehydes, 6 ketones, 4 carboxylic acids, 3 hydrocarbons, and 4 other compounds not included in the previous groups. The percentage of each compound, expressed as relative abundance, is reported in Table 3. Based on the HCA analysis, clustering the VOCs chemical classes with percentages of mycelial growth inhibition of all phytopathogenic fungi, three clusters of chitosan formulations were observed (Figs. 4, 5, 6, 7, and 8). The first cluster included CH1 and CH5 together with CH2, the second one contained CH7 and CH9 plus CH8, and the third one included CH3 and CH6 together with CH4.

In the first cluster, CH1, CH5, and CH2, the class of other compounds (CH1: 65.95 ± 0.96; CH5: 51.51 ± 0.79; CH2: 49.78 ± 0.66) was the most represented with a high presence of dichloromethane (CH1: 54.27 ± 0.81; CH5: 51.51 ± 0.79; CH2: 21.85 ± 0.19). Furthermore, only in CH1 and CH2 was recorded the presence of phenol, 2,5-di-tert-butyl, with the highest value in CH1 (11.68 ± 0.15). Aldehydes were found in CH2, but not in CH1 and CH5; whilst carboxylic acids were recorded only in CH5.

In the second cluster, aldehydes were the most represented chemical class in CH7 (43.72 ± 0.27) and CH9 (41.31 ± 0.32), followed by ketones (CH7: 20.98 ± 0.23; CH9: 25.49 ± 0.23). In CH7, the most abundant aldehyde was benzaldehyde (10.12 ± 0.05), followed by furfural (8.56 ± 0.07), nonanal (8.32 ± 0.03), and hexanal (7.23 ± 0.06), whereas 2-pentanone (5.32 ± 0.05) was its most abundant ketone. In CH9, the most represented aldehydes were benzaldehyde (9.55 ± 0.08), and furfural (9.26 ± 0.09) followed by nonanal (7.85 ± 0.04), and the main ketones were 2-pentanone (6.87 ± 0.05) and 3-hexanone (5.64 ± 0.05). Meanwhile, CH8 presented the highest level of ketones (27.23 ± 0.28) and hydrocarbons (15.50 ± 0.10) among the tested chitosan formulations, with a lower quantity of aldehydes (19.30 ± 0.11) compared to CH7 and CH9.

Considering the third cluster, in CH3 and CH6 VOCs profile the most represented chemical classes were aldehydes (CH3: 57.09 ± 0.52; CH6: 42.44 ± 0.41) followed by carboxylic acids (CH3: 23.22 ± 0.52; CH6: 23.91 ± 0.18). The most abundant aldehydes in CH3 profile were decanal (12.43 ± 0.14) and nonanal (12.42 ± 0.13), whereas nonanoic acid (22.24 ± 0.51) was the most abundant carboxylic acid. In CH6 profile nonanal (12.44 ± 0.14) and acetic acid (9.11 ± 0.07) were the most abundant aldehyde and carboxylic acid, respectively. In CH4 the most represented chemical class was carboxylic acids (65.33 ± 1.21), including octanoic acid (31.44 ± 0.57) and acetic acid (21.70 ± 0.49). A lower amount of aldehydes (7.89 ± 0.05) was recorded compared to CH3 and CH6. No hydrocarbons were found in VOCs profile of CH3 and CH6, whilst they presented significant amounts in CH4 (6.44 ± 0.11). These chitosan formulations presented low content in ketones and other compounds.

The HCAs run with the mean percentages of VOCs GC peak areas and the percentages of mycelial growth inhibition against the different postharvest pathogens, placed antifungal activity against *B. cinerea*, *M. fructicola*, *A. alternata*, and *A. brassicicola* in the same cluster with

Table 3
Identified individual VOCs and their GC peak areas obtained from the nine chitosan formulations at a concentration (w/v) of 1 % (CH1, CH2, CH3, CH4, CH5, CH6, CH7, CH8, and CH9). Results were obtained using static headspace solid-phase micro-extraction (HS-SPME) followed by gas chromatography coupled with mass spectrometry (GC-MS).

					Area (%) ± S.E.[1]								
Peak No.	**Compounds**	**R.T.**	**$RI^{2}_{Lit.}$**	**$RI^{3}_{Exp.}$**	**CH1**	**CH2**	**CH3**	**CH4**	**CH5**	**CH6**	**CH7**	**CH8**	**CH9**
	Ketones:												
1	2-Propanone	2.72	496	-	-	-	1.43 ± 0.04	-	-	-	2.37 ± 0.04	6.01 ± 0.06	3.84 ± 0.04
4	2-Pentanone	3.75	686	685	6.36 ± 0.07	1.13 ± 0.03	-	-	-	-	5.32 ± 0.05	7.54 ± 0.07	6.87 ± 0.05
5	2-Pentanone, 4-methyl	4.40	690	691	-	-	-	-	2.87 ± 0.05	-	3.71 ± 0.03	5.24 ± 0.04	0.86 ± 0.01
7	3-Hexanone	5.11	775	773	4.60 ± 0.05	2.82 ± 0.04	-	-	2.85 ± 0.05	-	4.15 ± 0.04	3.12 ± 0.04	5.64 ± 0.05
8	2-Hexanone	5.19	791	789	4.33 ± 0.06	2.27 ± 0.03	-	-	3.81 ± 0.04	-	2.31 ± 0.03	4.21 ± 0.05	3.14 ± 0.03
13	Ciclohexanone	7.59	895	894	-	-	-	0.77 ± 0.02	-	-	3.12 ± 0.04	1.11 ± 0.02	5.14 ± 0.05
	Total ketones				**15.29 ± 0.18**	**6.22 ± 0.10**	**1.43 ± 0.04**	**0.77 ± 0.02**	**9.53 ± 0.14**	**-**	**20.98 ± 0.23**	**27.23 ± 0.28**	**25.49 ± 0.23**
	Aldehydes:												
10	Hexanal	5.39	806	801	-	-	1.25 ± 0.02	-	-	5.45 ± 0.06	7.23 ± 0.06	2.15 ± 0.02	5.64 ± 0.03
12	Furfural	6.10	835	841	-	-	-	-	-	8.32 ± 0.06	8.56 ± 0.07	6.22 ± 0.03	9.26 ± 0.09
14	Heptanal	7.75	905	903	-	-	1.66 ± 0.02	-	-	1.88 ± 0.04	2.55 ± 0.01	0.85 ± 0.01	3.47 ± 0.04
15	Benzaldehyde	9.46	961	954	-	7.98 ± 0.06	-	0.34 ± 0.01	-	-	10.12 ± 0.05	4.11 ± 0.02	9.55 ± 0.08
16	Octanal	10.63	990	994	-	-	7.26 ± 0.04	-	-	6.56 ± 0.05	0.96 ± 0.01	-	1.11 ± 0.02
19	Nonanal	13.61	1089	1086	-	-	12.42 ± 0.13	-	-	12.44 ± 0.14	8.32 ± 0.03	2.41 ± 0.01	7.85 ± 0.04
20	(*E*)−2-Nonenal	15.18	1162	1159	-	-	0.89 ± 0.02	-	-	-	0.84 ± 0.01	-	-
21	Decanal	16.49	1175	1171	-	-	12.43 ± 0.14	-	-	7.79 ± 0.06	5.14 ± 0.03	3.56 ± 0.02	4.43 ± 0.04
23	3,4-Dimethylbenzaldehyde	16.88	1208	1189	-	14.08 ± 0.13	4.98 ± 0.03	7.55 ± 0.04	-	-	-	-	-
24	(*E*)−2-Decenal	18.05	1256	1258	-	-	8.67 ± 0.05	-	-	-	-	-	-
26	Undecenal	20.71	1308	1305	-	-	6.10 ± 0.03	-	-	-	-	-	-
	Total aldehydes				**-**	**22.06 ± 0.29**	**55.67 ± 0.48**	**7.89 ± 0.05**	**-**	**42.44 ± 0.41**	**43.72 ± 0.27**	**19.30 ± 0.11**	**41.31 ± 0.32**
	Carboxylic acid:												
3	Acetic acid	3.27	650	643	-	-	-	21.70 ± 0.49	22.38 ± 0.47	9.11 ± 0.07	-	-	-
22	Octanoic acid	16.75	1180	1182	-	-	-	31.44 ± 0.57	-	6.57 ± 0.06	1.26 ± 0.01	6.54 ± 0.04	2.33 ± 0.02
25	Nonanoic acid	19.36	1272	1281	-	-	22.24 ± 0.51	-	-	-	6.14 ± 0.05	1.14 ± 0.02	3.25 ± 0.03
27	Decanoic acid	21.12	1370	1381	-	-	0.98 ± 0.01	12.19 ± 0.15	-	8.23 ± 0.05	1.85 ± 0.01	-	-
	Total carboxylic acid				**-**	**-**	**23.22 ± 0.52**	**65.33 ± 1.21**	**22.38 ± 0.47**	**23.91 ± 0.18**	**9.25 ± 0.07**	**7.68 ± 0.06**	**5.58 ± 0.05**
	Hydrocarbons:												
9	Octane	5.33	800	799	-	-	-	3.44 ± 0.05	-	-	-	9.95 ± 0.06	0.64 ± 0.04
11	2,4-Dimethylheptane	5.79	819	825	-	-	-	2.14 ± 0.03	-	-	2.16 ± 0.02	-	-
17	Decane	11.31	1000	998	-	-	-	0.86 ± 0.03	-	-	0.54 ± 0.02	5.55 ± 0.04	0.59 ± 0.03
	Total hydrocarbons				**-**	**-**	**-**	**6.44 ± 0.11**	**-**	**-**	**2.70 ± 0.04**	**15.50 ± 0.10**	**1.23 ± 0.07**
	Others:												
2	Dichloromethane	2.84	501	499	54.27 ± 0.81	21.85 ± 0.19	3.49 ± 0.02	2.30 ± 0.02	51.51 ± 0.79	14.82 ± 0.31	5.13 ± 0.04	13.20 ± 0.37	7.21 ± 0.11
6	Propylene glycol	4.47	700	694	-	19.35 ± 0.35	-	-	-	-	-	-	-
19	Methyl benzoate	13.33	1078	1083	-	1.91 ± 0.04	-	-	-	-	5.55 ± 0.03	2.23 ± 0.02	3.41 ± 0.02
28	Phenol, 2,5-di-tert-butyl	24.20	1514	1511	11.68 ± 0.15	2.67 ± 0.02	-	-	-	-	-	-	-
	Total others				**65.95 ± 0.96**	**49.78 ± 0.66**	**3.49 ± 0.02**	**2.30 ± 0.02**	**51.51 ± 0.79**	**14.82 ± 0.31**	**10.68 ± 0.07**	**15.43 ± 0.39**	**10.62 ± 0.13**

[1] N = 3 replicates; [2] RILit= linear retention index from the literature; [3] RIExp= determined linear retention index against mixture of n-alkanes (C5–C40) on HP-5MS column

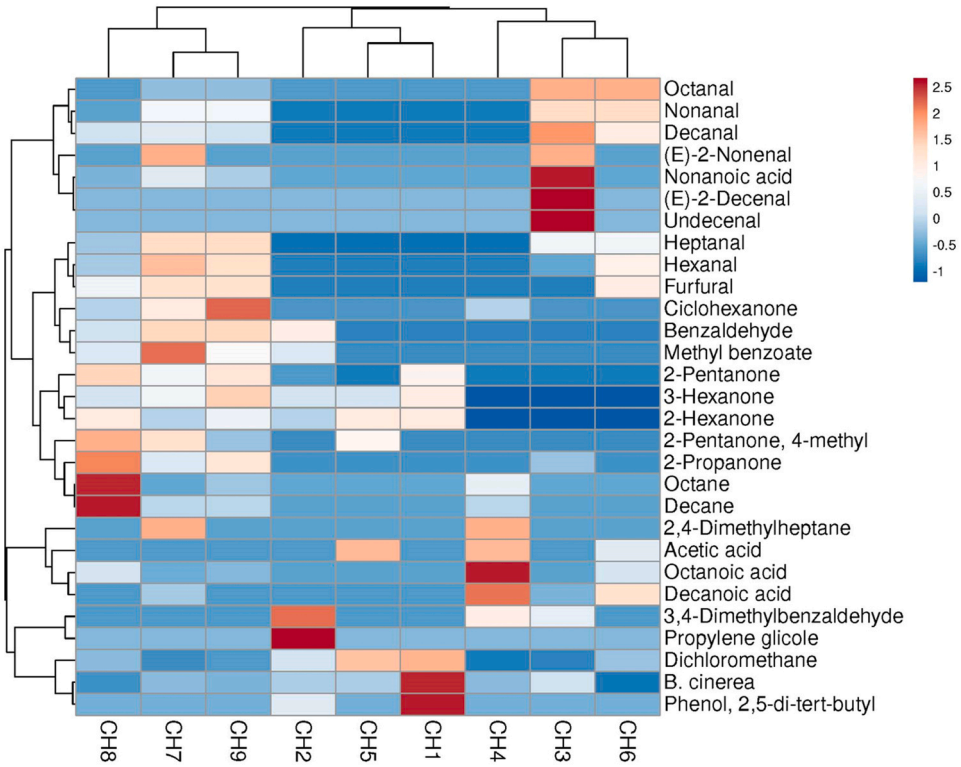

Fig. 4. Heat map from the hierarchical cluster analysis (HCA) grouping the nine chitosan formulations (1 % w/v) based on their similarity regarding GC peak areas individual VOCs identified and the percentages of mycelial growth inhibition (PI) against *Botrytis cinerea*. The color represents the relative abundance or level of each parameter, and the clusters show the grouping among the chitosan formulations (top part) and the parameters, either peak area or PI (left side).

phenol, 2,5-di-tert-butyl and dichloromethane (Figs. 4, 5, 7, and 8). Whereas mycelial growth inhibition of *M. laxa* resulted in the same cluster of 3-hexanone, 2-hexanone, and 2-pentanone plus dichloromethane and phenol, 2,5-di-tert-butyl (Fig. 6).

4. Discussion

Results from the present study demonstrate that chitosan formulations release VOCs with diverse chemical composition, which, in turn, cause differential effects on fungal growth. When present, these inhibitory effects seem to be dose dependent, since successive dilutions reduce their inhibitory activity.

Out of the nine tested formulations, only three of them presented consistent and significant antifungal activity against all tested fungal strains; namely CH1, CH7, and CH9. This suggests that the presence of growth-inhibitory VOCs in the chitosan formulations cannot be directly assigned to the chemical structure of their active principle (chitosan or chitosan derivative), nor to their source of origin. For example, the highly inhibitory formulation CH1, and non-inhibitory ones such as CH4 and CH5 have the same active principle (chitosan hydrochloride). In the same way, CH7 and CH9, which produced strong inhibition, present the same active principle (chitosan) than non-inhibitory ones like CH3, CH6, and CH8. Similarly, CH1 and CH4 on one hand; and CH7, CH8, and CH9 on the other, share the same source (crustaceans and mollusks, and *Aspergillus niger*, respectively) but differ in their antifungal activity. It worth highlighting the differences between CH7, CH8, and CH9, since they are different formulations of the same product, manufactured by the same company with the same active principle and source. All this strongly suggests that the presence and release of VOCs with biological activity from these products can be traced to additives or substances derived from their manufacturing and formulation process, and/or from compounds present in the original sources but that are differently affected or eliminated during the successive manufacturing steps. This was partly to be expected, given that pure chitosan alone, described as a soluble solid biopolymer with high MW (National Center for Biotechnology Information - NCBI, 2025) would not be an expected source of natural VOCs at environmental conditions, since it begins to decompose and produce volatile compounds such as pyrazines, pyridines, pyrroles and furans only at around 525 K , (Zeng et al., 2011).

In this context, assuming that the antimicrobial activity of VOCs is not directly correlated to the active principle in the formulations (chitosan or chitosan derivative) but to other compounds present in the tested products, it would be reasonable to assume that it is neither directly determined by the concentration of the said active principles. Therefore, it becomes difficult to draw accurate comparisons of effectiveness between the specific tested concentrations among the different formulations. We can compare the results of the formulations at 1 % concentration of active ingredient.

It is relevant to highlight that certain treatments seem to promote fungal growth, especially on *M. fructicola* (CH2, CH3, CH4, and CH5 at their highest concentrations). Previous studies have already reported growth promoting activity of VOCs on different fungal pathogens (Briard et al., 2016; Álvarez-Garcia et al., 2021; Marzouk et al., 2021). Therefore, further research on this aspect could be of interest to unveil the full potential of VOCs from chitosan formulations, as well as the possible risks they may pose.

Makau et al. (2023) previously demonstrated the efficacy of chitosan products in direct contact method, the result showed that COS-OGA (CH2) at concentrations of 1 % and 0.5 % completely suppressed mycelial growth of *A. alternata*, *A. brassicicola*, *B. cinerea*, *M. laxa*,

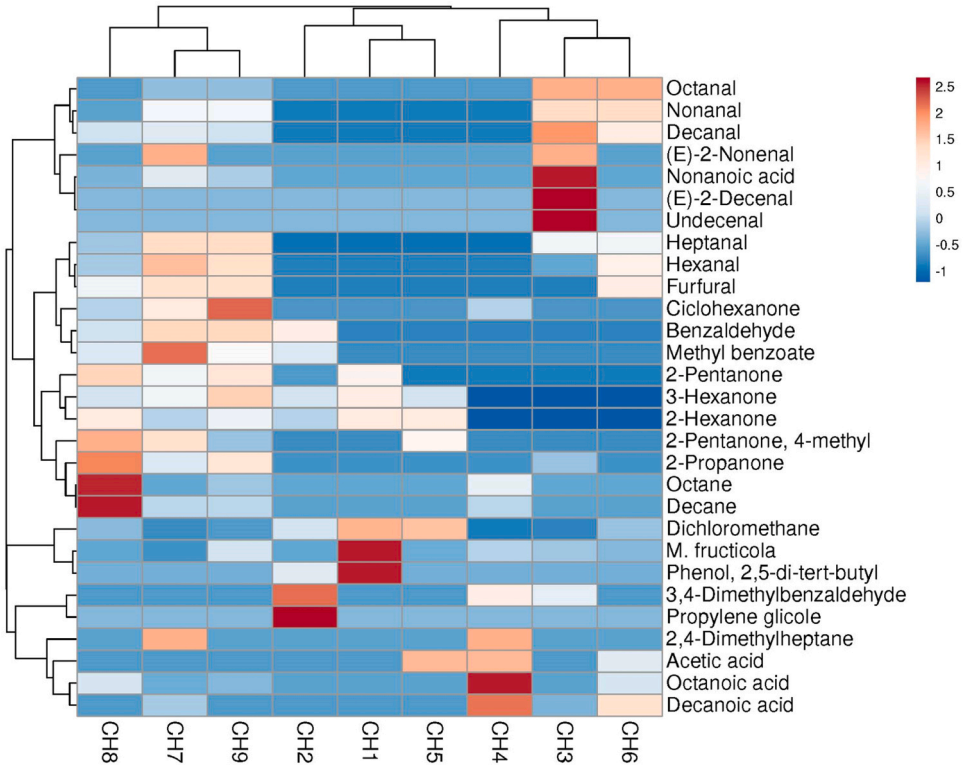

Fig. 5. Heat map from the hierarchical cluster analysis (HCA) grouping the nine chitosan formulations (1 % w/v) based on their similarity regarding GC peak areas individual VOCs identified and the percentages of mycelial growth inhibition (PI) against *Monilinia fructicola*. The color represents the relative abundance or level of each parameter, and the clusters show the grouping among the chitosan formulations (top part) and the parameters, either peak area or PI (left side).

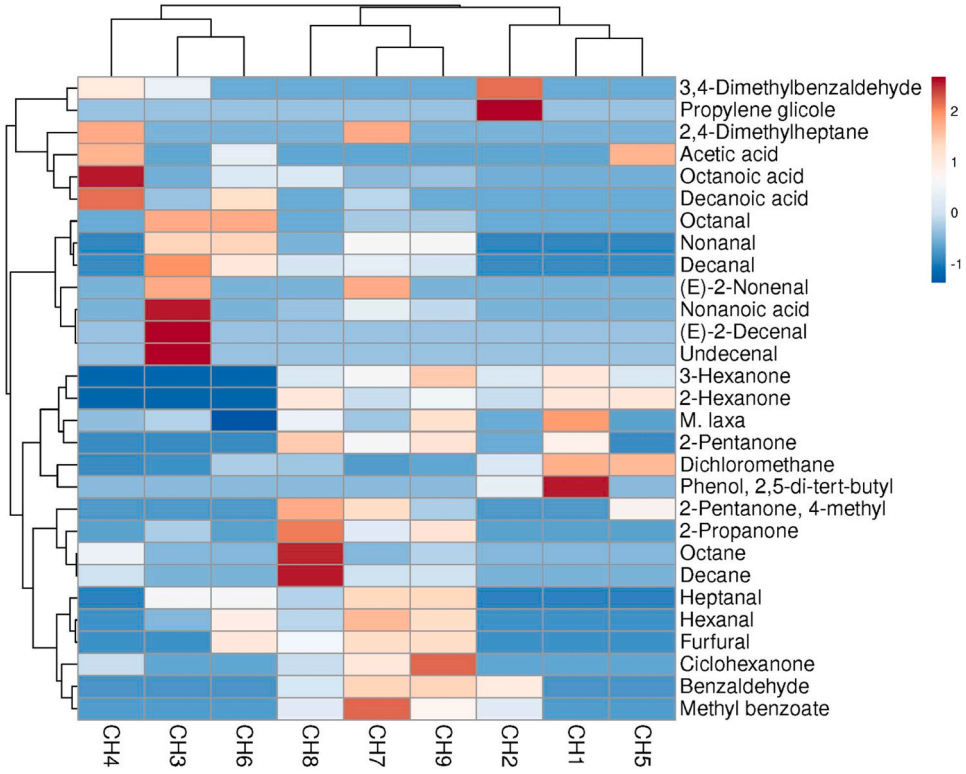

Fig. 6. Heat map from the hierarchical cluster analysis (HCA) grouping the nine chitosan formulations (1 % w/v) based on their similarity regarding GC peak areas individual VOCs identified and the percentages of mycelial growth inhibition (PI) against *Monilinia laxa*. The color represents the relative abundance or level of each parameter, and the clusters show the grouping among the chitosan formulations (top part) and the parameters, either peak area or PI (left side).

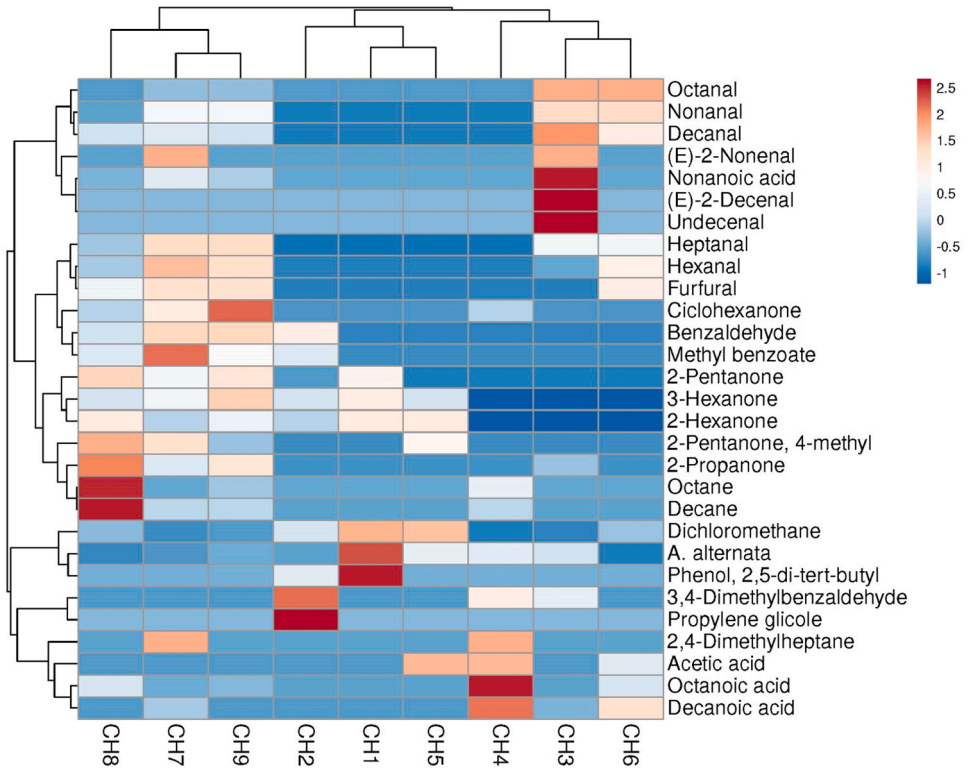

Fig. 7. Heat map from the hierarchical cluster analysis (HCA) grouping the nine chitosan formulations (1 % w/v) based on their similarity regarding GC peak areas individual VOCs identified and the percentages of mycelial growth inhibition (PI) against *Alternaria alternata*. The color represents the relative abundance or level of each parameter, and the clusters show the grouping among the chitosan formulations (top part) and the parameters, either peak area or PI (left side).

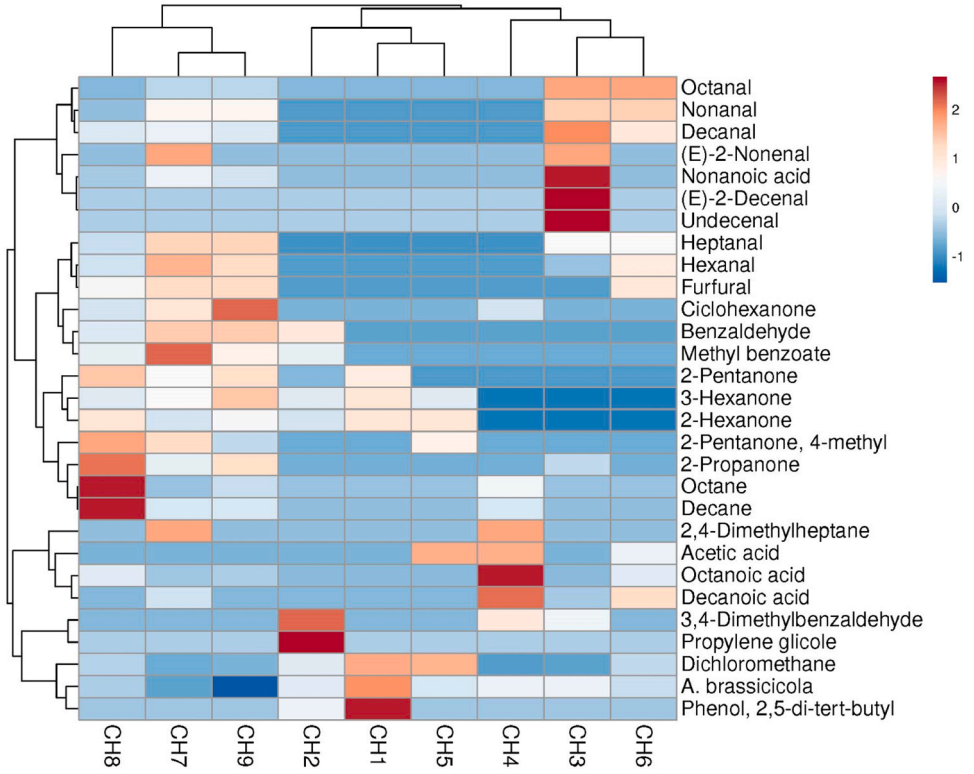

Fig. 8. Heat map from the hierarchical cluster analysis (HCA) grouping the nine chitosan formulations (1 % w/v) based on their similarity regarding GC peak areas individual VOCs identified and the percentages of mycelial growth inhibition (PI) against *Alternaria brassicicola*. The color represents the relative abundance or level of each parameter, and the clusters show the grouping among the chitosan formulations (top part) and the parameters, either peak area or PI (left side).

M. fructigena, and *M. fructicola*. Furthermore, chitosan hydrochloride at concentrations of 1–0.25 % effectively inhibited *M. laxa* and *M. fructigena*, while *M. fructicola* was susceptible to concentrations of 1–0.5 %.

To the best of our knowledge, there are no studies regarding either the VOCs directly released by chitosan formulations or their antifungal activity, other than those specifically used as VOC carriers. In this regard, Heras-Mozos (2022a) reported no differences concerning the antifungal activity of VOCs between controls with and without chitosan coating.

Attending to the individual VOCs released by the tested chitosan formulations, phenol, 2,5-di-tert-butyl was the molecule that presented the strongest correlation with their antifungal activity against *B. cinerea, M. fructicola, A. alternata,* and *A. brassicicola* and to a lesser extent against *M. laxa*. It was mainly detected in the headspace of CH1, and therefore it is the main candidate for its strong antifungal activity.

This compound, also named as 2,5-Di-tert-butylphenol; Phenol, 2,5-bis(1,1-dimethylethyl)-; or 2,5-bis (1,1-Dimethylethyl) phenol, is a phenolic compound and an alkylbenzene with the molecular formula $C_{14}H_{22}O$ and a molecular weight of around 206 Da (NCBI, 2024a). It has been previously described as a microbial metabolite produced by actinobacteria (Das et al., 2018), such as *Streptomyces malaysiense* (Ser et al., 2016). Culture supernatants from these strains presented antimicrobial activity (Das et al., 2018). It was also the major component in leaf extract from the plant *Zizyphus spina-christi*, which demonstrated significant antifungal activity against *A. alternata*, *Alternaria citri*, and *Alternaria radicina* (El-Shahir et al., 2022). It was also described in *Psidium guajava* root bark extracts with antiviral activity (Velmurugan et al., 2012). Moreover, ethyl acetate extract of *Persicaria strigosa* leaves showed a relevant amount of phenol, 2,5-di-tert-butyl as well as anthelmintic activity (Swargiary et al., 2021).

A similar molecule, Phenol, 2,4-bis(1,1-dimethylethyl)- was synthesized by lichen-associated *Streptomyces mobaraensis*, *Nocardiopsis synnemataformans*, and by *Fusarium venenatum*, being described to produce strong antimicrobial activity, although not in volatile phase (Vijayakumar et al., 2024). The same compound was produced by *Vibrio alginolyticus* and showed anti-quorum sensing and anti-biofilm activity against *Serratia marcescens* (Padmavathi et al., 2014).

Phenol, 3,5-bis(1,1-dimethylethyl)-, produced by the plants such as *Prunella vulgaris, Vernonia glabra*, *Ranunculus rionii* and *Ceratophyllum demersum* presented putative antifungal activity against *Fusarium oxysporum*, *Alternaria solani* and *Penicillium digitatum* (Akhtar et al., 2024), and potential antimycobacterial and antibacterial activity (Lupoae et al., 2016; Banda et al., 2022). The same molecule, produced by *Streptomyces* sp. and *Nocardia* sp. has been suggested to present a broad-spectrum antimicrobial activity against bacterial pathogens (Sharma et al., 2016; Konwar et al., 2023). Both Phenol, 2,4-bis(1,1-dimethylethyl)-; and Phenol, 3,5-bis(1,1-dimethylethyl)- isolated from *Lantana camara* presented also termiticidal activity (Patel and Narasimhacharya, 2024).

The antimicrobial activity of these compounds has been proposed to derive from their phenolic hydroxyl groups interacting with plasma membrane proteins and lipids through their electrophilic groups, leading to destabilization and damage of the cell membrane and potentially affecting cell respiratory processes (Konwar et al., 2023). Furthermore, Phenol, 2,4-di-tert-butyl has been proposed as an inhibitor against the siderophores Pyoverdines of *Pseudomonas aeruginosa* in *in silico* studies (Vijayakumar et al., 2024).

Some of these and other related compounds, namely Phenol, 2,6-bis (1,1-dimethylethyl)-; Phenol, 2,4-bis(1,1-dimethylethyl)-; and Phenol, 3,5-bis(1,1-dimethylethyl)- were detected in gut associated bacteria from the marine crab *Lissocarinus orbicularis* (Harikrishnan et al., 2021), which could be related with the presence of Phenol, 2,5-di-tert-butyl in CH1, since the source of this chitosan formulation are crustaceans and mollusks.

In the present study, dichloromethane (DCM) showed also correlation with the antifungal activity of the tested chitosan formulations according to the HCA. It is a highly volatile organochlorine compound with the formula CH_2Cl_2 and a molecular weight of around 85 Da, used extensively as a solvent in chemical manufacturing and research (NCBI, 2024b). Nevertheless, the antimicrobial effects of DCM alone are not explicitly mentioned in bibliography. Most studies focus on the antimicrobial properties of DCM extracts or fractions from various natural sources. These extracts or fractions are typically obtained by dissolving the biological material in DCM and then separating the resulting solution to isolate the bioactive compounds. The antimicrobial effects are attributed to these bioactive compounds present in the DCM extracts or fractions (Awan et al., 2023; Mcotshana et al., 2023; Mirahmad et al., 2024). The widespread use of DCM as solvent, as well as the fact that it is the only individual VOC identified in all 9 chitosan formulations, suggests that in the present study it could be a remnant additive from their manufacturing process. CH5, which demonstrated negligible antifungal activity, showed high levels of DCM, very similar to those of CH1, suggesting that this compound might not be crucial for the antifungal activity of the studied chitosan formulations.

Additionally, 3-hexanone, 2-hexanone, and especially 2-pentanone correlated with *M. laxa* mycelial growth inhibition. These ketones have been found to hold bioactive properties, such as insecticidal activity (Germinara et al., 2012), or inhibiting spore germination of *Clostridium botulinum* (Bowles and Miller, 1993). They are a common feature of microbial volatilomes, some of which have shown antifungal activity against *Fusarium solani* and *Sclerotinia sclerotiorum* (Petre et al., 2017), *F. oxysporum* (Asghar and Kataoka, 2025), and *Cladosporium inversicolor* (Huang et al., 2021), among others. No previous information has been found relating these ketones to the growth inhibition of *Monilinia* spp.

Final mention deserve the compositional differences between CH7, CH8, and CH9. As stated, they are different formulations of the same product, with the same active ingredient and source. In this context, while CH7 and CH9 presented significant antifungal effects against all strains, CH8 only slightly inhibited *M. laxa*. The main differences between them regarding VOC composition point to aldehydes. CH7 and CH9 presented higher levels of total aldehydes, as well as cyclohexanone, in comparison to CH8, which suggests that some of these compounds may explain part of the differences in antimicrobial activity among them.

The presence of potentially toxic substances, such as DCM and phenolic compounds rises some concerns regarding both human health and environmental damage, which should be addressed in further studies considering individual toxicant levels in the formulations and their putative deleterious effects. For instance, DCM has shown hepatotoxic effects in animal models via inhalation and it is classified as likely to be carcinogenic in humans, both in liver and lungs (Schlosser et al., 2014). Meanwhile, phenolic compounds such as phenol, 2, 5-di-tert-butyl cause skin and respiratory irritation, severe eye irritation and may cause long lasting harmful effects to aquatic life (NCBI, 2024a).

5. Conclusions

This study demonstrates that VOCs released by chitosan formulations present diverse chemical composition and produce differential growth inhibitory effects on the fungal pathogens *B. cinerea*, *M. fructicola*, *M. laxa*, *A. alternata*, and *A. brassicicola*. This volatile bioactivity seems to be dose dependent, although not directly linked to chitosan concentration, its chemical nature, or source; thus, pointing to other factors such as the manufacturing process and formulation. CH1, CH7, and CH9 were the most active formulations against the tested fungi. Results from the HCAs suggested a strong correlation between dichloromethane and, especially, phenol, 2,5-di-tert-butyl with the antifungal activity observed against all five tested pathogens. Whereas *M. laxa* mycelial growth inhibition correlated also with 3-hexanone, 2-hexanone, and 2-pentanone. This work opens a new road for further research and

characterization of VOCs from chitosan formulations, their biological activities, and their potential use in pre and postharvest disease management, as well as their possible effect on plant metabolism, plant defense, microbiome, and the environment. Additionally, this work highlights the importance of coformulants, which can provide a further contribution in the overall effects. The work opens a new window in the multiple applications of chitosan, introducing novel effects that can potentiate its use in the preservation of fresh fruit and vegetables by postharvest decay and consequent reduction of loss and waste of fresh produce.

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CRediT authorship contribution statement

D'Isita Ilaria: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Pistillo Onofrio Marco:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Álvarez-García Samuel:** Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Romanazzi Gianfranco:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Moumni Marwa:** Writing – review & editing, Validation, Software, Investigation, Formal analysis, Conceptualization. **Germinara Giacinto Salvatore:** Writing – review & editing, Supervision, Methodology, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

Data will be made available on request.

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