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Essential oil-based emulsions reduce bacterial canker on kiwifruit plants acting as antimicrobial and antivirulence agents against *Pseudomonas syringae* pv. *actinidiae*

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Abstract

Pseudomonas syringae pv. *actinidiae* (Psa) poses a significant threat to global kiwifruit production, with current control measures proving insufficient and fostering resistance development. Essential oils (EOs) offer a promising alternative due to their multifaceted antimicrobial and antivirulence mechanisms. This study evaluated the antimicrobial activity of various EOs—cinnamon bark (CIN), oregano (ORE), clove bud (CLO), and thyme (THY)—against Psa, in terms of growth and virulence traits. CIN exhibited the highest antimicrobial activity, followed by ORE and CLO EOs, while THY EO was less effective. Encapsulation of EOs into organic polymer-based emulsions enhanced their antimicrobial efficacy by improving bioavailability and stability while reducing the required dosage. Notably, CIN and ORE EO emulsions effectively reduced disease symptoms in kiwifruit under both in vitro and in vivo conditions. Mechanistically, these EOs demonstrated dual activity: direct antimicrobial effects likely via membrane alteration and indirect antivirulence effects, including the inhibition of biofilm production and type III secretion system induction. Field trials further confirmed the potential of EO-based formulations to reduce disease incidence and severity over a growing season. This study underscores the potential of EO emulsions as sustainable, cost-effective plant protection agents, aligning with the goals of environmentally friendly crop management strategies.

Keywords Bacterial disease management, Natural compounds, Antimicrobial activity, Type III secretion system inhibition, Biofilm inhibition, Field application

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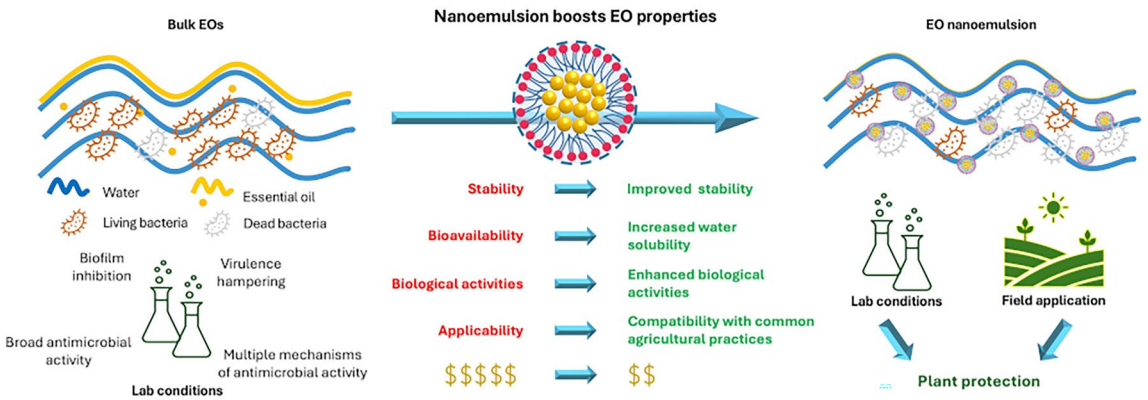
Graphical Abstract



Background

Bacteria have been recognized as plant pathogens for over a century, and since then, the management of plant bacterial diseases has relied mainly on copper-based compounds in Europe [33, 54]. Copper compounds are widely used in the field due to their stability, methods of action, and water solubility, as well as because they also provide protection against fungal pathogens [33].

Bacterial resistance to copper compounds is particularly relevant for perennial crops, where bacteria survive through different growing seasons, and the population adapts over time [33]. Such resistance relies on the presence of genetic features, some of them carried and transmitted thanks to mobile genetic elements, thus a reservoir of genome-borne resistance can also be found in non-pathogenic bacteria [23, 24]. Copper resistance is frequently found in plant pathogenic bacteria, like within the *P. syringae* complex, which is also characterized by a long-range diffusion due to its association with clouds and rainfall [45]. The worldwide distribution and broad polyphagy of *P. syringae* make this species particularly interesting to monitor since it developed resistance against copper and antibiotics. A rapid evolution of resistance occurred in *P. syringae* pv. *actinidiae* (Psa), the causal agent of kiwifruit bacterial canker, a major threat to kiwifruit cultivation worldwide since its first isolation in 1984 [63]. Indeed, while no copper-resistant strains of Psa were reported before 2010, from 2014, some strains were found to be resistant to copper in New Zealand as well as in countries where the cultivation of kiwifruit is important [14, 16, 45]. The rapid evolution of antimicrobial resistance in Psa highlights the need to find alternatives to current bacterial control products that do not push the evolution of resistance mechanisms but hamper pathogen virulence [19].

Psa is divided into 5 biovars (Psa 1, Psa 2, Psa 3, Psa 5 and Psa 6), which differ in their virulence and seem to be diffused within a restricted region, with the exception of Psa3 [15, 22, 38]. Psa biovar 3 (Psa3) is the most aggressive biovar and was responsible for the severe pandemics of 2008 in Italy and 2010 in New Zealand [21, 22]. Psa3 does not produce toxins, but it is characterized by the rapid activation of the type three secretion system (T3SS) when incubated in apoplast-like conditions [61] and is highly responsive to the presence of kiwifruit-derived signals [47]. It has been proposed that this likely contributes to Psa3 aggressiveness, since the ability to secrete effectors into the host is a pivotal step in its colonization, and it is considered a major virulence factor within the *P. syringae* complex [2]. Indeed, *P. syringae* mutants defective in effector secretion are unable to infect the host and the suppression of the T3SS results in a really attenuated pathogen [18, 34, 37], making this virulence mechanism a valid target for reducing bacterial diseases. Quorum sensing (QS) regulates an aspect important for Psa survival in the environment, i.e., the ability to form biofilm, which can protect the colony from external threats and chemicals.

These aspects strengthen the idea of developing crop protection strategies aiming to inhibit pathogen growth and T3SS-mediated virulence simultaneously. Among the new strategies to control bacterial plant pathogens, a few can address both those aspects independently and, in this context, EOs play a major role. Indeed, EOs are attracting huge interest due to their multifaceted ability to restrict microbe growth and their broad range of activity [11, 49]. EOs are a complex mixture of several compounds, and

some of them carry a biological activity, which can be displayed as bactericidal/fungicidal, virulence attenuator, QS mechanism inhibitor as well as plant defence inducer [32, 49]. Some EOs are recognized as GRAS (generally recognized as safe) in USA by the Food and Drug Administration (FDA) and are generally safe for humans when used in small amounts [71]. The antimicrobial activity of EOs is exerted through the permeabilization of microbe membranes, the destabilization of cellular processes, the interference with cell-to-cell communication and QS [3, 27, 53]. The antimicrobial activity of EOs or their principal active substances against *P. syringae*, and specifically against Psa, was previously reported [10, 42, 55] (Danzi et al. in press). Regarding virulence inhibition, although not reporter in Psa, it was recently shown that linalool, a monoterpene present in diverse EOs, reduces the expression of the *hrpA* gene, encoding the main T3SS pilus, in *P. syringae* pv. *tomato* DC3000 [17]. These studies focused on the in vitro activity of EOs showing their potential to control bacterial growth and/or virulence mechanisms but the application of EOs in the field is not straightforward since they are highly susceptible to hydrolysis and UV irradiation, which can impair their activity [58]. These drawbacks can be circumvented by encapsulating EOs within organic matrices that can concurrently protect EOs from degradation and increase their dispersion in water. Moreover, organic polymers have a reduced environmental impact with respect to their inorganic counterparts and could be easily available [39]. Indeed, polymers like alginate and chitosan could derive from agricultural or organic waste, likely reducing the production cost [39]. The encapsulation of EOs is considered to be effective in protecting them from degradation and keeping their antimicrobial activity besides enabling their dispersion in water [36].

In a recent work, EOs of CIN, CLO, ORE and THY were evaluated for their antimicrobial activity against Psa in terms of growth inhibition and their further encapsulation in chitosan-based emulsions, providing promising results about the possibility of applying these new formulates to manage kiwifruit bacterial canker (Danzi et al. 2024). However, the evaluation of emulsion stability showed that it flocculates when diluted in tap water. Considering that the aim is to propose alternative strategies applicable in the field, this aspect was considered a key limitation for further product development. Here, we describe the development of new emulsions containing EOs encapsulated into another organic polymer, namely alginate. Such a formulation increased EO antimicrobial activity and improved their dispersion in water, leading to a reduction of the disease index both in controlled and field conditions. Moreover, the study not only provides information about the physiochemical properties of the EO-based emulsions, but also knowledge about EO mode of action, including a direct antimicrobial effect associated with an antivirulence activity. This is, to our knowledge, one of the few example of studies reporting the activity of encapsulated EOs in open field against a bacterial plant pathogen. The results obtained here are thus encouraging to foster the development of this strategy to control kiwifruit bacterial canker and, likely, more broadly, bacterial diseases.

Materials and methods

Bacterial strain and culture conditions

Cryogenic stocks of *Pseudomonas syringae* pv. *actinidiae* CRAFRU 8.43 (hereafter Psa) [21] were streaked on solid King's B medium (KB) and grown at 28 °C for 48 h. To obtain fresh liquid cultures of Psa, single colonies were inoculated in liquid KB medium and incubated overnight at 28 °C with constant shaking.

Production and characterization of EO emulsions

All EOs used in this study were purchased from FLORA s.r.l. (Lorenzana-Pisa-Italia). The chemical compositions of the major constituents for each EO are reported in Tables 1–4 (Supplementary Tables 1–4). Emulsions of EOs were prepared by pre-homogenization of the polymer (sodium alginate) and the surfactants (soy lecithin) in water at high speed (6000 rpm) for a few seconds in homogenizer (Ultra-Turrax T25, IKA). Then, the EO was added to the mixture and homogenized at 25,000 rpm for 4 min. The emulsions produced were stored at room temperature, avoiding exposition to light. For physicochemical property characterization, emulsions (after production or storage) were diluted 1000 times in MilliQ water prior to be subjected to size and ζ-potential characterization (data are provided as Supplementary Figure S1). Size analysis of emulsions was performed by dynamic light scattering; emulsions were diluted to 1% in water and equilibrated at 25 °C for some minutes. Five measurements with at least 12 runs were taken for each sample, then measures were averaged and shown as intensity. Similarly, for ζ-potential measurements, emulsions were equilibrated at 25 °C, then at least 3 measurements with 10 runs per measure were taken, and then results were averaged. For accelerated ageing tests; after production, an aliquot was placed at 54 °C for 14 days while another was placed at 0 °C for 4 days. The viscosity of CIN and ORE EO-based emulsions was evaluated using a DSR 500 CP4000 rheometer (Lamy Rheology Instrument) equipped with the MK-SV418 spindle. Measurements

were taken under a constant shear rate of 150 Hz for 180 s at a constant temperature of 25 °C.

Evaluation of bacterial membrane permeabilization

From an inoculum of Psa grown overnight in KB at 28 °C under constant agitation, a bacterial suspension was prepared at OD 0.1 in HG buffer (Hepes 5 mM, Glucose 5 mM pH 7.2) after washing the inoculum 3 times in the same buffer. EOs were added to the as-prepared inoculum at the following concentrations 0.1%, 0.05%, 0, 025% for CIN EO, 0.33%, 0.165%, 0.0825% for ORE EO, 1%, 0.5%, and 0.25% for THY EO, and finally 0.25%, 0.125%, and 0.0625% for clove EO. All the higher concentrations reflect, for each EO, the MIC value at which complete growth inhibition is observed. Suspensions with the above concentrations of EOs were prepared in a 96-well plate in a final volume of 200 µl. 1.5 µl of 1 mM propidium iodide was added to each well. The plates were incubated in the dark for 20 min and then the fluorescence value was read at 617 nm (excitation at 535 nm).

Evaluation of *hrpA1* gene promoter induction

From an inoculum of Psa carrying the pBBR1-MCS5-p*hrpA1*::*gfp*^{C} reporter system [62] grown overnight in KB at 28 °C under constant agitation, a bacterial suspension was prepared at OD 0.1 in HIM [26] using glycerol as the only carbon source. In this experiment, EOs were used at concentrations well below the MIC so as to avoid any inhibition of bacterial viability, namely 0.05%, 0.025%, 0.0125%, and 0.006125%. Raw kiwifruit leaf extract at 1% was also added to the bacterial suspension to ensure the optimal activation of the *hrpA1* gene promoter [47]. The experiment was set up in a 96-well plate so as to evaluate all treatments simultaneously. Fluorescence readings at 535 nm (excitation at 485 nm) were evaluated at regular 15-min intervals for a total of 5 h.

In vitro evaluation of EO and emulsion antimicrobial activity in liquid medium

Psa was grown overnight, and then bacterial cells were diluted to OD_{600} 0.02 in KB medium. The bacterial suspension was distributed in a 96-well microtiter plate, and then EOs or EO emulsions were added to reach the desired final concentration. Emulsions were diluted in sterile MilliQ water before use and added directly to the wells. Plates were placed in a microplate reader (Tecan Infinite®200 Pro) with constant agitation (180 rpm) at 28 °C. Absorbance (OD_{600}) values were recorded for 24 h at regular intervals of 1 h. Bacterial growth was calculated as the ratio between the test condition's absorbance and the control's absorbance (no treatment) after subtracting the value measured at the beginning of the experiment. To estimate the vitality of bacteria, 16 µL of INT ((2-(4-iodophenyl)−3-(4-nitrophenyl)−5-phenyl-2H-tetrazolium, Sigma Aldrich) previously prepared at the final concentration of 1 mg/ml in MilliQ water, were added to each well. The plate was placed in agitation at room temperature for 20 min until the color of the control wells turned red. Three independent biological replicates were performed with a minimum of 3 technical replicates for each condition. The growth percentage was calculated as already described, and data from the biological replicates were averaged. An ANOVA analysis followed by a Tukey post hoc test (p-value 0.05) was performed to assess the statistical differences among the different conditions.

The evaluation of the antimicrobial activity of the combination of different EO emulsions was performed likewise. At the end of the experiment, the concentrations at which no growth was observed were considered as the minimum inhibitory concentrations (MIC). Then, the calculation of the fractional inhibitory concentration index was performed as follows:

$$\begin{aligned} \mathrm{FICI} &= \mathrm{FIC_A} + \mathrm{FIC_B} \\ &= (\mathrm{C_A}/\mathrm{MIC_A}) + (\mathrm{C_B}/\mathrm{MIC_B}), \end{aligned}$$

where MIC_A and MIC_B are the MICs of the EOs A and B alone, and C_A and C_B are the concentrations of the EOs in combination.

The combination is defined as synergistic when the FICI is equal or less than to 0.5; additive FICI is greater than 0.5 and smaller/equal to 1.0; FICI is indifferent when greater than 1.0 and smaller/equal to 4.0; and antagonistic if FICI is greater than 4.0.

Biofilm inhibition

Bacterial suspensions were prepared as described in the previous section and were finally dispensed in MBEC® (Innovotech) 96-well plates. EO emulsions were added to the bacterial suspension at the beginning of the experiment. Plates were then incubated at 28 °C in constant agitation (180 rpm) for 24 h. Then, the lid was removed and washed three times in sterile 0.9% NaCl and placed into a new 96-well plate filled with the same solution. The plate was tightly sealed and placed into a bath sonication for 30 min to allow the removal of the biofilm from the pegs. The suspension obtained was serially diluted to allow colony count on nutrient agar plates (Oxoid™). Colony count was performed also to estimate the planktonic growth.

In vitro protection assay on kiwifruit plants through flood-inoculation

Flood inoculation of Psa was performed following the procedure described in Ishiga et al. [28], with some adaptations. Plantlets of *Actinidia chinensis* var. *deliciosa* Hayward K5 were purchased from a local supplier (Vitroplant) and aseptically transferred into a sterile box containing half-strength Murashige and Skoog (MS) medium containing vitamins, 1% sucrose and 0,7% Gelrite. Psa was inoculated by flooding plants with a bacterial suspension at OD_{600} 0.02 diluted in distilled water containing 0.025% of Silwett L-77. Plants were submerged with the bacterial suspension for 5 min, and, after suspension removal, the boxes were placed in a growth chamber at 18 °C with a photoperiod of 12 h. Symptom development was monitored for 3 weeks after the inoculation of Psa. EO emulsions were applied 24 h before or after bacterial inoculation. The disease index was calculated as the percentage of leaves showing symptoms out of the total number of leaves. The severity index was estimated according to a scale from 0 to 8, with 0 corresponding to the absence of spots, 1 corresponding to 1 necrotic spot, 2 corresponding to 2–3 necrotic spots, 3 corresponding to 4–5 necrotic spots, 4 corresponding to more than 6 necrotic spots, and 5–6–7–8 corresponding to the presence of symptoms on 25–50–75–100% of the leaf surface, respectively (Fig. S6B). Bacterial count was performed by grinding kiwifruit leaves with $MgCl_2$ 10 mM and then tissues were homogenized for 1 min at 3000 rpm using a TissueLyser (Qiagen). The supernatant was recovered and serially diluted with sterile $MgCl_2$ 10 mM. Finally, drops of 10 µL were placed onto KB-agar plates and let dry under sterile laminar flow. Plates were incubated in the dark for 36–48 h and then the colony count was performed.

Protection assay in the field

Field evaluation was performed in a local orchard of *A. chinensis* var. *chinensis* (Faenza-Ravenna-Italy). The emulsion containing ORE or CIN EO were individually applied at the concentration of 2% (CIN and ORE), and in combination (50% CIN and 50% ORE) at the concentration of 2% (MIX). Emulsions were applied, after blooming, once per month for four consecutive months from May to September 2023. Three trees from three different rows were chosen as samples, obtaining a total of nine trees per treatment. The parameters observed to evaluate the presence of symptoms caused by Psa was the disease index (ratio of symptomatic leaves to total leaves per plant) by calculating for each plant the number of infected leaves of three secondary branches. The severity index was calculated based on the score represented in Supplementary Fig. S6C. Symptoms were evaluated the week after treatment application.

Statistical analyses

All experiments, but in the field, were performed three times with at least three technical replicates each. The results presented in the different figures represent the average of the biological replicates and the statistical significance was calculated using the one-way ANOVA ($p < 0.05$) followed by a Tukey post hoc test, unless otherwise stated.

Results

EOs increase *Psa* membrane permeability

Membrane permeability is an important stress indicator for bacteria since their damage can cause the leakage of nutrients and invalid metabolic processes, leading ultimately to cell death [7]. The ability of selected EOs to increase membrane permeability was tested in vitro using the propidium iodide as an indicator since it is a non-permeable DNA stain able to reach the cytosol only when membranes are damaged or, at least, more permeable [35]. All EOs, used at their reported MIC values according to Danzi et al. (2024, in press), led a significant fluorescence signal, indicative of PI binding to bacterial DNA, proportional to EO concentration. These results demonstrate a dose-dependent permeabilization of the membranes in presence of the EOs, showing that bacterial cells are heavily compromised by the treatments (Fig. 1). Thus, all EOs exploit cell membrane alteration, at least partly, for their antimicrobial activity. Of note, although the most active among the selected EOs, CIN EO triggered the lowest fluorescence signal at its highest concentration, reported to inhibit completely Psa growth in vitro, suggesting that this may not represent the predominant mechanism of CIN EO on Psa (Fig. 1A).

EOs reduce *hrpA1* promoter activity in *Psa*

The ability of EOs to inhibit the type III secretion system (T3SS) in Psa was assessed using a reporter system allowing to monitor the activity of the promoter of the *hrpA1* gene, encoding the major pilus of the structure [62]. This system has been demonstrated as a good indicator of the attenuation of T3SS function and thus the virulence of Psa [47, 61]. To avoid any effect on EOs on bacterial vitality, which may account for a reduction of *hrpA1* promoter activity, EOs were used at concentrations that do not interfere with Psa growth. For all the tested EOs, the highest concentration used (i.e., 0.05%) inhibited Psa *hrpA1* promoter activation by 80–100% compared

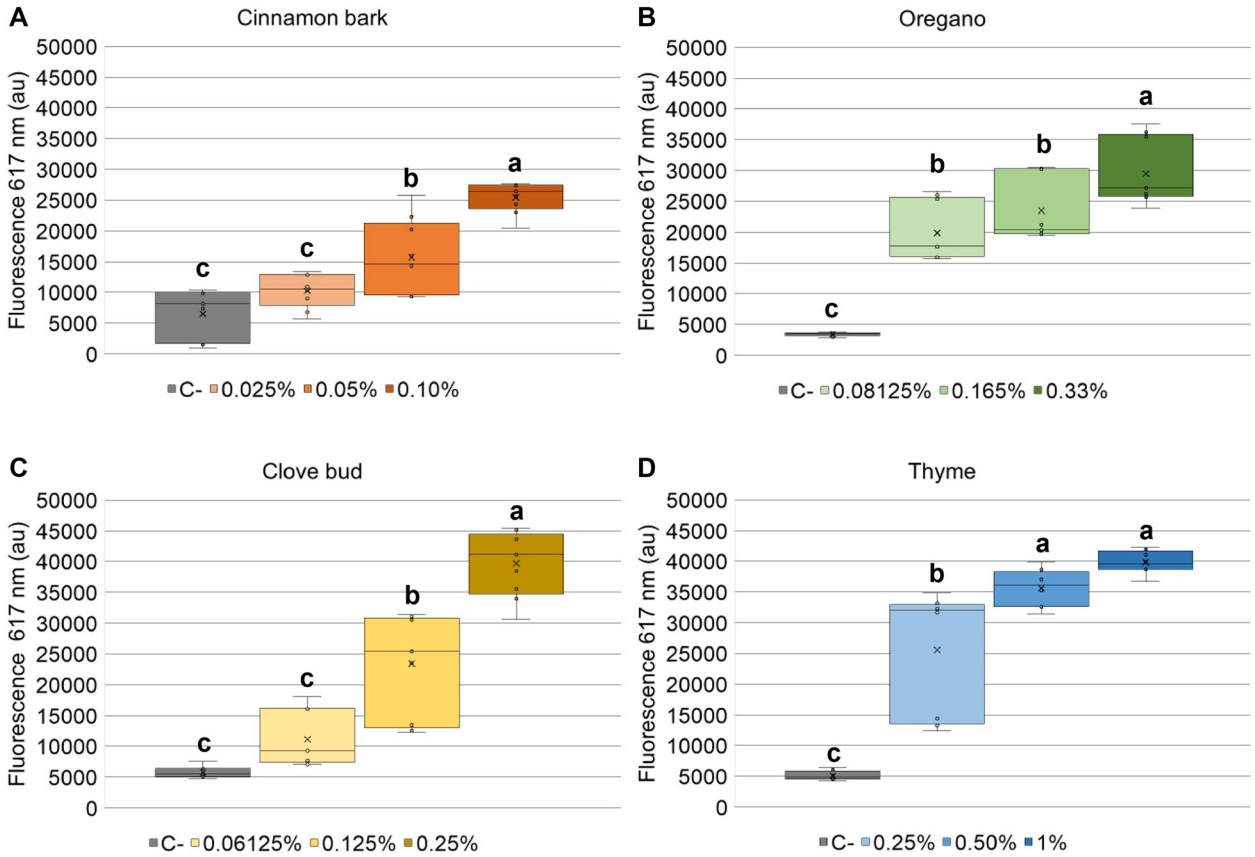


Fig. 1 Effect of essential oils on the membrane permeability of *Psa*. Psa CRAFRU8.43 cells were treated with different concentrations (as indicated) of essential oils, namely **A** CIN, **B** ORE, **C** CLO, and **D** THY before being incubated with propidium iodide (PI). As a negative control, cells were treated with H_2O. The different letters indicate statistically significant differences among the samples

to the control (Fig. 2). Surprisingly, for all EOs but CLO, such inhibition was no more observed at concentrations below 0.05%, suggesting the existence of a concentration threshold below which the efficiency is lost. By contrast, there was a dose-dependency trend for CLO EO, although not statistically significant due to the variability of the response (Fig. 2C). Overall, these results show that, though EOs display a diverse degree of antimicrobial activity (different MICs), they have all the ability to inhibit similarly the activation of the T3SS.

EOs emulsions display heterogeneous droplet sizes and a high stability

The emulsions of the EOs were produced to obtain formulations that are effective, stable over time and showing a low environmental impact. To this end, oil-in-water (O/W) emulsions were produced using alginate as the organic polymer and lecithin as an organic surfactant to encapsulate the EOs and to improve their water solubility. Eventually, a stabilizing agent was added to the formulation.

The emulsions were characterized for their size and surface charge through optical microscopy and dynamic light scattering (Supplementary Fig S1A-C). CIN EO-based emulsions showed the highest particle size, having an average diameter of 317.3 nm (± 81.37 nm; Supplementary Fig. S1A, peak 1), while ORE and CLO EO-based particles reached 241.6 (± 102.1 nm; Supplementary Fig. S1B peak 1) and 251.2 nm (± 116.5 nm; Supplementary Fig. S1C peak 1), respectively. The high value of the standard deviation of particle size reflected the presence of heterogeneous particle populations. Nevertheless, the main peak represented 96.1%, 99.4% and 99.0% of particles produced with CIN, CLO and ORE EOs, respectively. In addition, a smaller sub-population of particles, represented by a short peak, was observed at 49.35 nm for CIN EO (3.9% of particle population) and 47.37 nm for ORE EO (1% of particle population). Conversely, a peak occurred at 4884 nm in CLO EO-based emulsion, indicating that very large particles were formed during the emulsification process, though representing

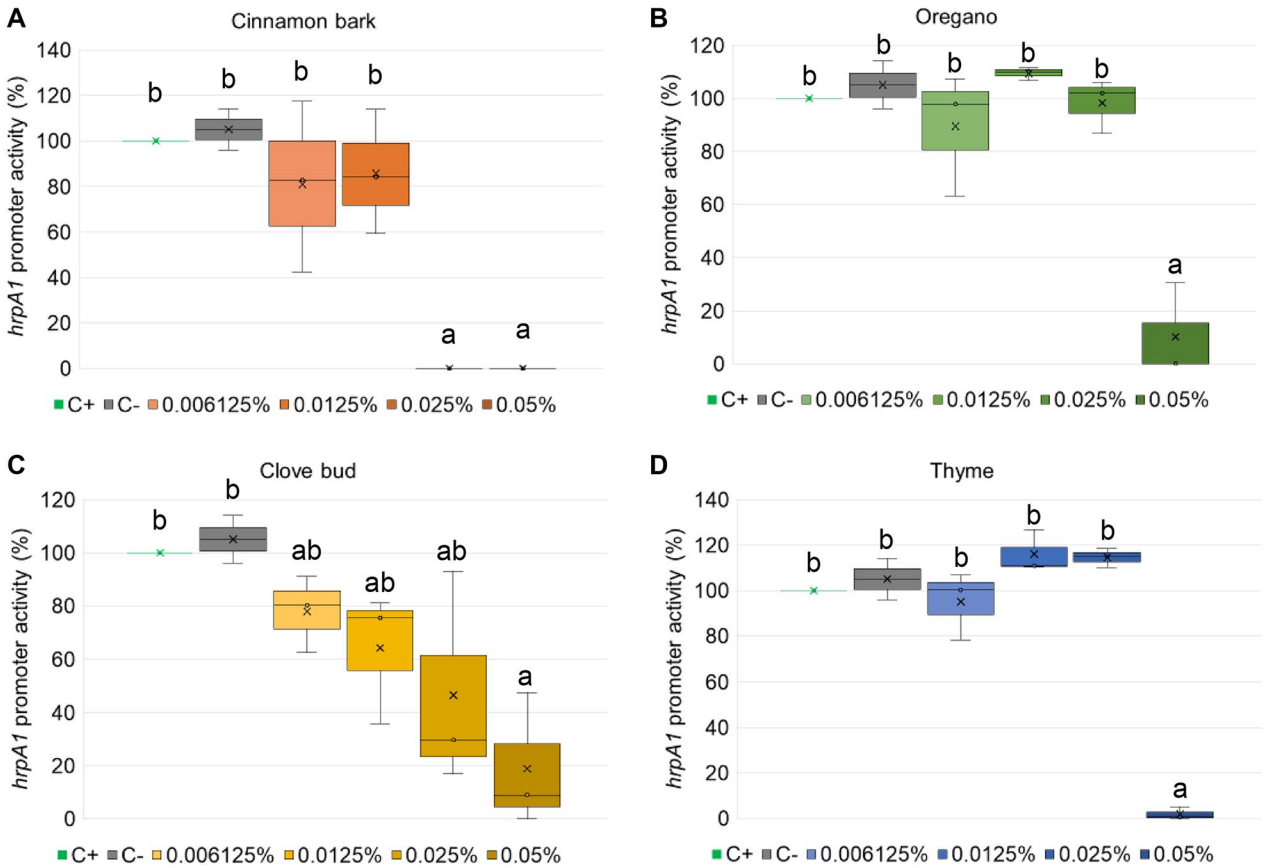


Fig. 2 Effect of essential oils on the induction of the type III secretion system of *Psa*. Psa CRAFRU8.43 cells carrying the p*hrpA1*::*gfp*^{C} reporter system were treated with different concentrations (as indicated) of different essential oils, namely **A** CIN, **B** ORE, **C** CLO, and **D** THY. Fluorescence was measured after 4 h of incubation and expressed as the % of promoter activation respect to the negative control (cell treated with H_2O), representing the maximal induction (100%). The different letters indicate statistically significant differences among the samples

only 0.6% of particle population. The emulsion produced could be considered thermodynamically stable since the Z-potential reached values well below −30 mV (Supplementary Fig. S1D). Of note, neither the addition of stabilizing agents nor the conservation at different temperatures modified significantly particle stability, thus confirming that the emulsions showed a good degree of stability per se. Finally, CIN and ORE EO emulsions showed a viscosity of 8.93 mPa.s^{-1} ± 0.27 and 2.76 mPa.s^{-1} ± 0.27, respectively (Supplementary Fig. S1E). Since smaller droplets (20–200 nm) lead to a lower viscosity compared to droplets > 200 nm, the slightly lower value observed for ORE EO could be attributable to its minor average droplet diameter compared to CIN EO (241.6 nm vs. 317.3 nm). Nevertheless, considering that below 10 mPa.s^{-1} emulsions are considered very fluid with water-like consistency, these results overall support EO suitability for further foliar spray applications.

EOs emulsions improve EOs antimicrobial efficacy against *Psa*

The EO-based emulsions were further evaluated for their ability to inhibit Psa growth in vitro. At a final EO concentration of 0.1% (v/v), a complete growth inhibition was observed for emulsions carrying CIN or ORE (Fig. 3A, B). Although not complete, the inhibition also reached more than 90% in the presence of CLO EO-based emulsion (Fig. 3C). It is worth mentioning that we did not test higher concentrations of this emulsion since this would strongly increase its cost of production thus not allowing further applications in crop protection. Finally, an almost complete inhibition of the growth was also observed for the CIN EO-based emulsion at 0.05%, but with a greater variability compared with 0.1%. Overall, the results highlight that the emulsion outperforms the antimicrobial activity of all the tested EOs with a 2- to threefold MIC reduction following EO encapsulation, though less evident for CIN. Such efficacy improvement was observed regardless of the presence of the stabilizing

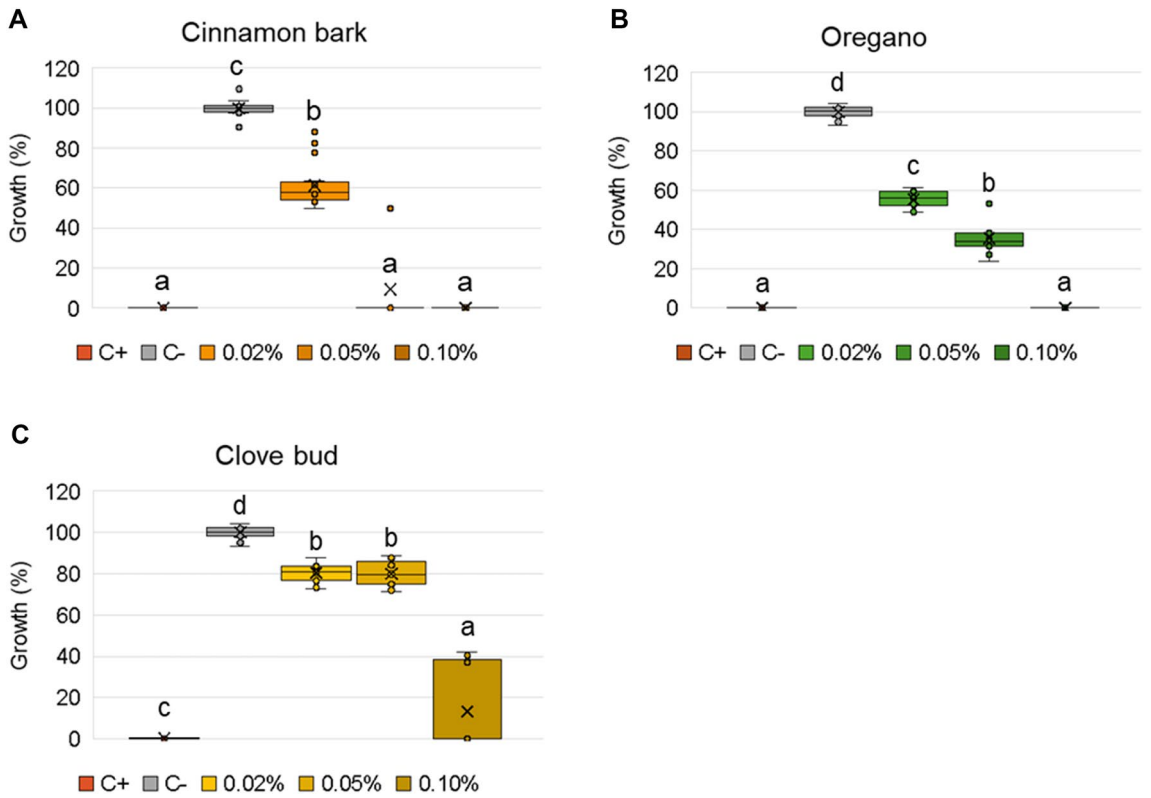


Fig. 3 In vitro antimicrobial activity of essential oil-based emulsions against Psa. Psa CRAFRU8.43 cell growth was measured over 15 h following treatment with different concentrations (as indicated) of emulsions obtained with **A** CIN, **B** ORE or **C** CLO essential oils. As controls, cells were treated with kanamycin (positive control; C +) or H_2O (negative control; C –). The different letters indicate statistically significant differences among the samples

agent in the emulsions produced with CIN and ORE EOs, when applied at concentrations below the MIC (Supplementary Fig. S2A-B). Conversely, a slight increase in growth inhibition was found for the clover bud-based emulsion supplemented with $CaCl_2$ or chitosan (Supplementary Fig. S2C). This is likely due to the lower efficacy of clover bud compared with the other EOs tested.

Of note, the alginate-based emulsions showed a similar antimicrobial activity compared with the previous formulation using chitosan as main polymer (Danzi et al., 2024 in press; Supplementary Fig. S3), thus justifying their employment since they are fully stable in tap water. Moreover, the emulsions maintained their antimicrobial activity after conservation at 0 °C in accelerating ageing tests (Supplementary Fig. S4). However, the test at 54 °C led to a decrease in emulsion efficacy on Psa growth, indicating that the formulations should be stored at a cold temperature to keep the full efficacy of the products.

Essential oil-based emulsions differentially affect biofilm production by Psa

To get further insights into the mechanism of action of the EO-based emulsions, they were evaluated on the production of biofilm by Psa using the Minimum Biofilm Inhibition Concentration (MBIC) assay (Fig. 4). To that purpose, an intermediate EO concentration of 0.075% was included for ORE and CLO, for which the MIC was fixed below 0.05%. Conversely, since such concentration was already bactericidal for CIN EO, this intermediate concentration was omitted. Regarding the planktonic population, in line with the results described above on liquid growth curve, both CIN EO- and ORE EO-based emulsions showed a predominant bactericidal activity, with a significant decrease of bacterial density with respect to the beginning of the assay at the highest tested emulsion concentrations, from 0.05% and 0.075%, for CIN and ORE, respectively (Fig. 4A, B). On the opposite, CLO EO emulsion showed mainly a bacteriostatic activity, i.e., almost no significant increase of the bacterial population respect to the t0 at all tested concentrations, except 0.025% at which a very low antimicrobial activity was noted (Fig. 4C). The evaluation of the inhibition of biofilm formation was then considered only starting from these bacteriostatic concentrations (indicated in red in Fig. 4D–F) to avoid any biofilm reduction due to cell

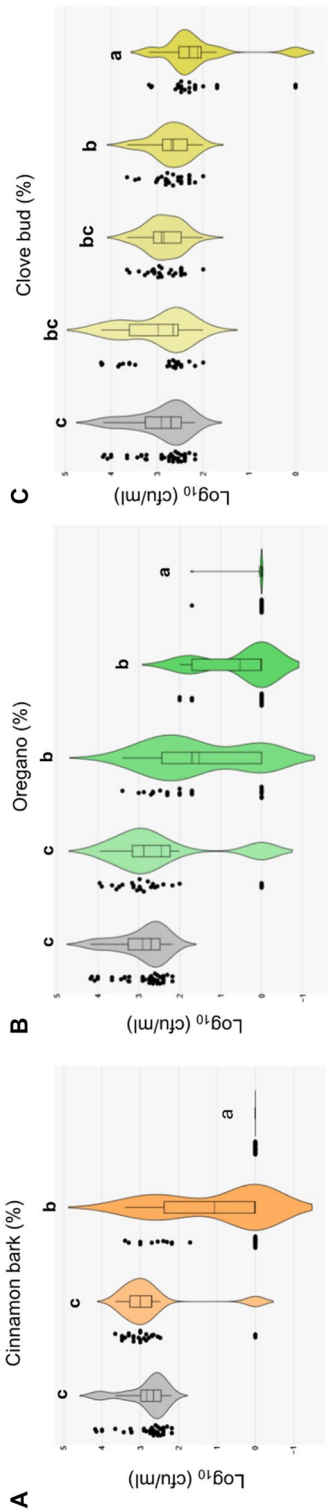


Fig. 4 Effect of essential oil-based emulsions on the formation of biofilm by Psa. Psa CRAFRU8.43 cells were incubated in presence of different concentrations of emulsions obtained with **A** CIN, **B** ORE or **C** CLO essential oils to determine the minimal biofilm eradication concentration (MBEC). The different letters indicate statistically significant differences among the samples. The asterisks indicate the concentrations, which are not bactericidal according to planktonic cell counts (Supplementary Figure S5)

killing. Only the formulation containing ORE EO was shown to strongly reduce biofilm formation (Fig. 4E). At the EO concentration of 0.05%, the number of bacteria within the biofilm matrix was reduced by 1.45 $\log_{10}$ cfu/ml, which corresponds to a reduction of almost 50% with respect to the control. On the other hand, though statistically significant, CLO EO caused only a weak biofilm reduction of around 0.25–0.5 $\log_{10}$ cfu/ml (Fig. 4F). Interestingly, despite the CIN EO emulsion possessing the highest antimicrobial activity among the selected EOs, it did not compromise the biofilm biomass when applied at a bacteriostatic concentration (Fig. 4D). This suggests that CIN EO targets solely the cells growing in the planktonic form and this opens the possibility to mix the EOs to combine their activities.

The blending of essential oil-based emulsions results in additive effects

The emulsions carrying different EOs were then combined to evaluate a possible improvement of their performance in terms of antimicrobial activity. All the combinations of two different EO emulsions, at 0.05% or 0.1%, resulted in an increase of Psa growth inhibition (from orange to purple; Fig. 5). The calculation of the fractional inhibitory concentration index (FICI) for each combination further indicated the combinations displayed an additive effect of the EOs ($0.5 < FICI < 1$), regardless of the combination of emulsions. Therefore, at least on Psa growth, the amount of EO seems more important than its composition.

The treatment with EO emulsions reduces disease severity in controlled conditions

Since the combination of ORE and CIN EOs was the most effective in restricting the growth of Psa in vitro and considering that ORE EO is also able to restrict biofilm production, this mix was exploited for the in planta assays and compared to the emulsion containing only CIN EO. The efficacy of EO emulsions in terms of protection against bacterial infection was evaluated using the flood-inoculation method and monitoring symptom development over 3 weeks. To reduce the surface tension on the leaves, tween 80 was added to the emulsions just before spraying on plants. The symptoms on kiwifruit plants appeared clearly from the first week following pathogen inoculation in absence of emulsion treatment (Supplementary Fig. S5) and eventually on the treated plants (Fig. 6). Indeed, the majority of the plants treated with the emulsions remained symptomless regardless of the treatment and the time of application (i.e., pre- or post-infection). These differences were further enhanced the second week after Psa inoculation, especially for the plants subjected to the preventive treatment (i.e., 24 h prior to infection; Fig. 6A, B). While the symptoms developed evenly on all plants, those treated with either the emulsion containing only the CIN EO or those containing an equal amount of both ORE and CIN EOs, showed lower disease and severity indexes (Fig. 5). However, as the disease progressed, although the trend remained, the statistical differences were almost abolished three weeks after pathogen inoculation, in particular when the treatments were applied in a protective manner (i.e., 24 h post-infection; Fig. 6C, D). Interestingly, the bacterial population in kiwifruit leaves did not change significantly among the different treatments, suggesting that Psa was able to colonize the tissue no matter the treatment is concerned, but lost its ability to induce virulence (Fig. S7A).

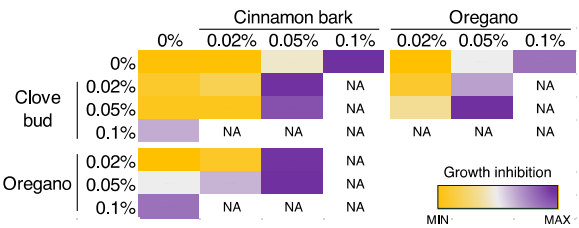


Fig. 5 In vitro antimicrobial activity of blended essential oil-based emulsions against Psa. Psa CRAFRU8.43 cell growth was measured over 15 h following treatment with different concentrations (as indicated) of emulsions obtained with CIN, ORE or CLO EOs, alone or in combination. Color intensity indicate the level of bacterial growth inhibition, from low (yellow) to high (purple) inhibition. The experiment was performed three times with 3 technical replicates for each condition. NA, not analyzed

EO emulsions protect kiwifruit plants from Psa infection in the field

Since data obtained in controlled conditions do not always consistently reflect the results in the field, the protection capacity of EO-based emulsions was finally evaluated in a kiwifruit orchard infected with Psa. One month after the first emulsion application (June), the disease index was lower for all the treatments compared with the control (not treated with EO-based emulsions) (Fig. 7). This trend was maintained throughout the growing season, as evidenced by a lower cumulative disease index at the end of the experiment, which was reduced by at least 20% with respect to the control (Fig. 7). Although the emulsion containing the ORE EO did not display the highest antimicrobial activity, it led to the lowest cumulative disease index. These data that confirm the protective capacity of EO-based emulsions are in accordance with the results obtained in vitro in controlled conditions.

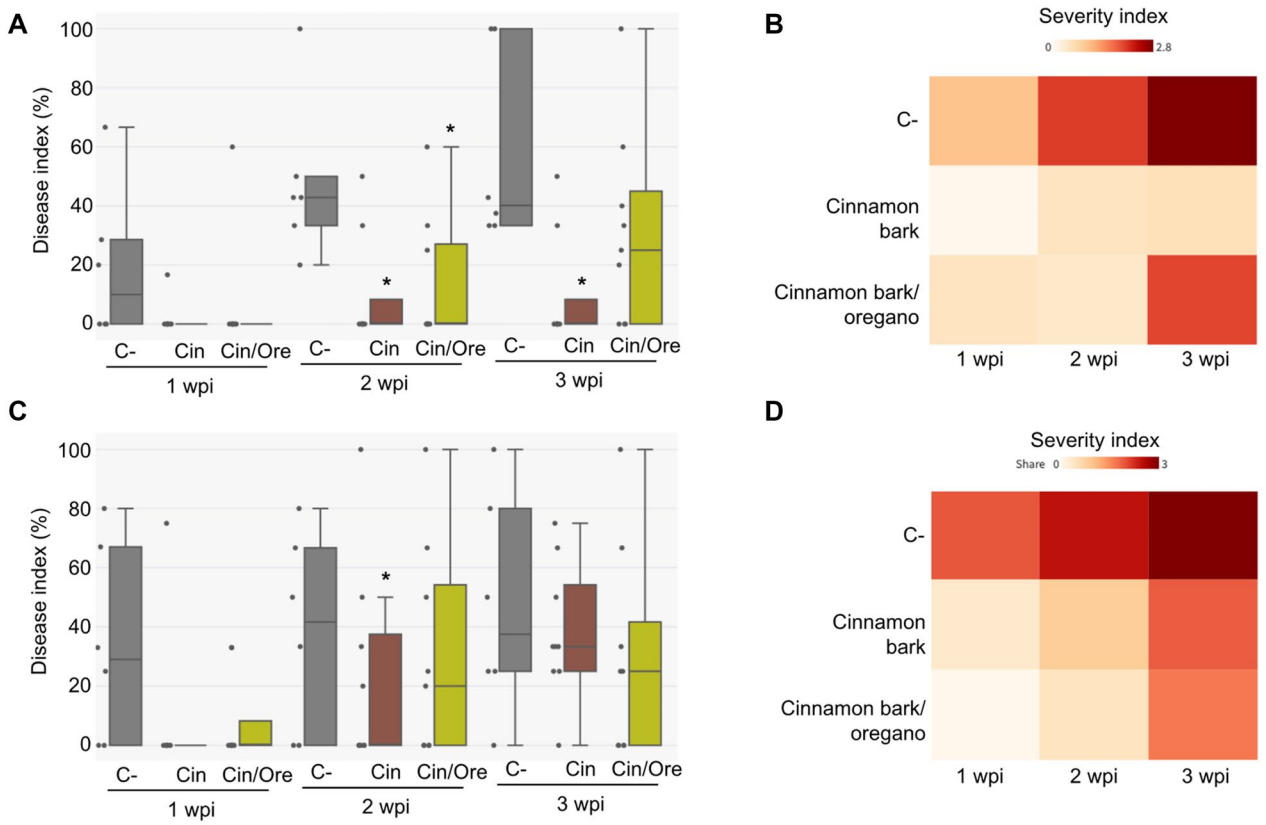


Fig. 6 Effect of essential oil-based emulsions on the appearance and development of bacterial canker symptoms in kiwifruit plants in vitro. In vitro-cultivated kiwifruit plants were infected with Psa CRAFRU8.43. The treatments with CIN essential oil-based emulsion, alone or blended with ORE essential oil-based emulsion, were applied 24 h prior to infection (**A, B**) or 24 h after infection (**C, D**). Disease index (**A, C**) and disease severity (**B, D**) were assessed weekly for 3 weeks. Plant treatment with H_2O was used as a negative control (C **—**). The experiment was performed three times including 9 plants for each condition in each biological replicate. The box plots represent the disease index of one representative biological replicate after 1, 2 and 3 weeks in pre- (**A**) or post- (**B**) infection. The asterisks indicate statistically significant differences respect to the negative control according to the Student's test. C-, negative control; Cin, treatment with CIN essential oil-based emulsion; Cin/Ore, treatment with blended CIN and ORE essential oil-based emulsions

Discussion

Psa is a major threat to kiwifruit production worldwide, and control strategies are not fully effective and have led to the occurrence of resistant strains [63]. The need for alternatives to control bacterial plant pathogens are currently driving researchers to develop products that provide long-lasting protection against pathogens. Thus, the development of a new class of agrochemicals should pursue target both pathogen growth and virulence through a variety of mechanisms and, in this context, EOs provide an interesting alternative due to their broad and multifaceted mode of action.

In line with the literature, the selected EOs possess high antimicrobial activity against Psa with a MIC in the range of 0.1% to 0.5% (v/v), CIN and ORE showing the highest ones. The pronounced antimicrobial activity of CIN EO against Psa was previously observed through a vapor diffusion assay [55]. Indeed, cinnamaldehyde, the main component of CIN EO, is a volatile compound that possesses a strong antimicrobial activity against a broad variety of microorganisms [55, 57, 64]. Similarly, the antimicrobial activity of ORE EO against bacterial pathogens is also widely recognized, and it was previously shown to be particularly effective against different strains of *P. syringae*. However, MIC values are highly variable among the *P. syringae* species, from around 3 to 12 mg/ml depending on the pathovar [10, 51]. Carvacrol exerts its antibacterial effect by destabilizing the bacterial cell membrane. In fact, it can intercalate within the phospholipid chains of the bacterial membrane, increasing its permeability [9]. However, the cell membrane is protected by lipo- and exopolysaccharides (LPS and EPS); thus, the differential susceptibility of *P. syringae* pathovars to ORE EO may be due to a differential composition of EPS. CLO EO is mainly composed of eugenol, which possesses a high antimicrobial activity previously

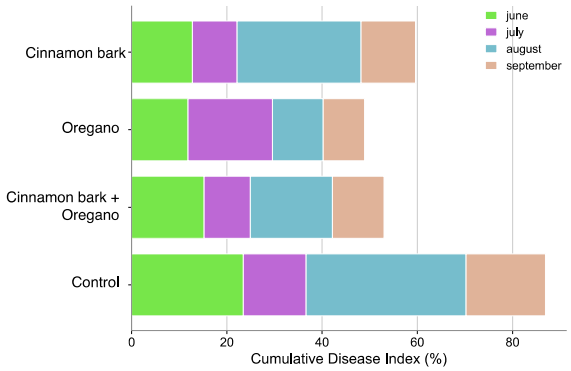


Fig. 7 Effect of essential oil-based emulsions on the progression of bacterial canker in kiwifruit orchard. Kiwifruit plants were treated with essential oil-based emulsions (CIN, ORE or blended emulsions; as indicated) at a final concentration of 0.2%, 35 days (8 June 2023), 65 days (6 July 2023), 95 days (8 August 2023) and 125 days (18 September 2023) after full bloom. Symptom development was monitored 8–10 days after treatment. Disease progression is represented as a cumulative disease index (% of leaves showing KBC symptoms during the season)

reported also against Psa [59]. Here, CLO EO showed a similar activity compared with ORE EO, but a lower ability to interfere with biofilm formation. Finally, THY EO was the least effective in inhibiting Psa growth in vitro, as reported in other *P. syringae* strains [10]. This low efficiency is likely due to the reduced amount of thymol, the principal antimicrobial compound present in this EO. Thymol possesses an antimicrobial activity very similar to that of its isomer carvacrol, in which the hydroxyl group is positioned in ortho (o-) instead of -meta (m-) with respect to the methyl group [4, 30]. However, its abundance is 20.7% in THY EO, while carvacrol reaches 70.31% in ORE EO; and this likely contributes to the difference in terms of antimicrobial activity between these two EOs. Similarly, *p*-cymene, the main component of THY EO (26.7%), showed a very reduced antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* in comparison with carvacrol [65]. The reduced activity was firstly attributed to the lack of hydroxyl group compared to carvacrol [20, 60], but it seems more likely that the poor antimicrobial activity of *p*-cymene may be related to its immiscibility in water and bacterial growth medium, thus lowering its bioavailability in conventional antimicrobial assays [65]. Consistently with this hypothesis, we observed a slight improvement of the antimicrobial activity on in vitro Psa growth of THY EO when encapsulated in a nanoemulsion (Danzi et al. 2024, in press). However, the activity remained insufficient to satisfy application requirements in term of efficacy, EO amount and associated costs (Danzi et al. 2024, in press). Thus, considering that the major component of THY EO (i.e., *p*-cymene) may possess little, if any, antimicrobial activity against Psa, even encapsulated, and thymol is present only in a low amount, THY EO is not an ideal candidate for developing an effective plant protection product against this bacterial phytopathogen, and was thus not included for further encapsulation.

All EOs were shown to interact with bacterial membranes, causing an increased permeability. The perturbation of membrane permeability is likely the most known mechanism by which EOs exhibit antimicrobial activity [3, 49]. CIN EO is the most effective in preventing Psa growth. Moreover, the interaction with membranes is supposed to be the first target of cinnamaldehyde (main component). Nonetheless, CIN EO led to the lowest membrane permeability alteration. Thus, the strongest antimicrobial activity of this EO could target predominantly bacterial cellular metabolism, involving different mechanisms occurring both on the cell surface and within the cytoplasm, and/or be dependent on the target microorganism [41, 64].

No difference in bacterial population was observed in in planta assays performed on kiwifruit in controlled conditions, despite symptoms were significantly reduced. Several components of essential oils, such as carvacrol [56], linalool [1, 17], *p*-cymene (Ahmad et al. 2024), thymol (Ahmad et al. 2024), eugenol [50, 72], cinnamaldehyde [6, 72], have been shown to interfere with the quorum sensing systems of pathogenic bacteria. The effects on the quorum signaling systems also reduce their virulence, including the formation of biofilm [5, 8, 31, 56, 70] and the assembly of secretion systems. More in detail, T3SS, a key virulence mechanism in Psa3 aggressiveness [61], is inhibited by the EOs assessed in this work. This evidence corroborates the idea that the tested EOs have a synergic mode of action: at high dosages there are antimicrobial, whereas at low dosages they are able to impair Psa virulence by altering biofilm formation and/or T3SS. Though EO-based emulsion concentrations applied on plants should have exerted an antimicrobial activity, based on in vitro data, we can assume that the effective concentrations within plant tissues were lower, thus shifting to an antivirulence activity that correlated with less severe symptoms in plants. This agrees with the emerging idea of developing agrochemicals that are less prone to being overcome by antimicrobial resistance, although their role as alternatives or substitutes to the classic protection strategies is still under debate. The employment of virulence-targeting crop protection products could indeed have a better environmental

impact, since they could target only pathogens on virulence traits, for instance carried by the T-PAI absent in commensal/beneficial bacteria [2]. EOs are also known to hamper QS-related bacterial traits [71], in phytopathogens like *Pectobacerium carotovorum* subsp. *brasiliense* and *carotovorum* [25, 29] and *Erwinia amylovora* [72], or plant-associated bacteria like *Pseudomonas fluorescens* [67]. Regarding the mode of action of the EOs on QS, carvacrol and eugenol exert their negative effect through their direct binding to the LuxI and LuxR proteins of *P. carotovorum* subsp. *brasiliense* [29], and, in the same way carvacrol reduces the secretion of polysaccharides and motility in *P. fluorescens* through the direct interaction with LuxR proteins [67]. Moreover, it cannot be ruled out that the negative QS regulation by EOs could also contribute to the observed T3SS inhibition, as demonstrated in *Salmonella typhimurium* and *Acidovorax citrulli* [66, 73]. Psa lacks a canonical QS system but it possesses three LuxR solos likely involved in intra- and interspecies as well as interkingdom communication [12, 13, 43]. Though their role in biofilm formation, and even more so in T3SS regulation, is still not fully elucidated, with possible positive and negative functions of PsaR1 and/or PsaR3, further investigations would deserve attention to figure out whether one or more component(s) of EOs may interact with Psa LuxR solos, leading to biofilm production and T3SS downregulation.

EO encapsulation into an organic polymer overcomes water solubility issues, using either chitosan (Danzi et al. 2024, in press) or alginate (this work). The innovative formulation with alginate presents a substantial advantage in comparison with chitosan-based formulation. In fact, it does not need a pH correction prior its application to plants, as chitosan, whose solubility at neutral pH is low. The analysis on the different EO emulsions by dynamic light scattering showed that particle size varies within the 240–320 nm range, thus depending also on the EO used. Such differences should be attributed to the chemical–physical properties, namely viscosity, molecular structure and concentration of volatile compounds of the EOs, that influence the size of particles in emulsions [52].

The emulsions produced were stable over time when stored at room temperature, while they showed small differences in the Z-potential after accelerated ageing tests, although the values were still within the stability zone, which considers that particles repulse if their Z-potential is higher than |30| mV [40]. However, emulsions lost some antimicrobial activity when stored either at high or low temperatures during the accelerated ageing assays, mimicking a storage period of 2 years. Storing EO emulsion at relatively high temperatures facilitates the loss of volatile active compounds, thus reducing emulsion efficacy. EOs are prone to degradation processes, particularly temperature and oxygen availability, which can induce modifications to their chemical structure [58]. The rise in temperature accelerates chemical reactions like that of hydroxyls, while low temperatures increase oxygen solubility and lead to the oxidation of functional groups [58]. It must be noted that introducing a stabilizing agent did not affect the antimicrobial activity or the stability of the emulsion. Thus, emulsions of these EOs can be produced without a stabilizing agent, reducing the overall cost of the product.

Emulsion increased the performance of EOs in terms of antimicrobial activity since MIC values were at least halved for all EOs. Since none of the co-formulants of the emulsion has an antimicrobial activity, this increase in antimicrobial activity is ascribable to a better essential oil bioavailability [46].

Since EOs can display different activities according to the bacterial species, mixing more EOs could increase their performance in inhibiting pathogen growth and virulence. Here, an additive effect was observed between emulsions carrying different essential oils. Although this does not translate into a better performance of the mixture with respect to individual EOs, it would be possible to combine different EOs to reduce the emulsion production cost. Indeed, EOs are expensive due to their extraction process, and the price can also vary in response to plant material availability. The combination of ORE and CIN EOs was the most effective in restricting the growth of Psa, and its activity was also maintained during in vitro and in vivo assays performed on plants.

All data obtained correlate positively with those obtained during kiwifruit infection in vitro. Indeed, emulsions proved to be effective in restricting Psa, especially during the early stages of infection. Both the incidence and the severity index were lower after emulsion treatment, regardless of the timing of their application. Interestingly, no difference in terms of bacterial population was observed, while the reduction in symptom appearance was evidenced in kiwifruit leaves. There is a little knowledge about the fate of EOs once they reach plant tissues besides their partitioning into a "soluble" and a volatile part [68]. EOs are thought to accumulate into the plant cell wall, but most of them are lost after application; thus, ensuring their attachment and permanence on leaves is of uttermost importance. Once inside the plant tissues, EOs can trigger defence responses, thus exploiting a double activity, directly against pathogens and indirectly by stimulating the transcription of defence-related genes [44, 48, 69]. The vapor phase of ORE EO induced the transcription of many defence-related genes as well as genes involved in hormone biosynthesis after 24 h

from treatment [51]. This treatment provided protection against *Plasmopara viticola* infection, demonstrating that the antimicrobial effect of EOs could be carried out through either direct or indirect mechanisms, although the net contribution remains unclear [51]. Therefore, the rate of protection given by EO-treatment is likely a combination of direct and indirect mechanisms where the latter's contribution depends on the host.

Conclusion

With this work, we evaluated the possibility of employing EOs for the protection of kiwifruit from Psa. EOs and their encapsulated formulations within an organic polymer displayed both antimicrobial and antivirulence activities against the bacterial pathogen, likely through distinct mechanisms, leading to plant protection in vitro. Indeed, the results highlighted a severe inhibition of disease severity in the model plant *A. thaliana*, while in kiwifruit the effect was milder but still significant. Finally, the in-field trial further supported CIN and/or ORE EO-based emulsions as a very promising strategy to be applied for kiwifruit protection, with a reduction of the cumulative disease index over a season. Though further investigations are required to optimize treatment conditions (timing, application number, doses) for protection improvement, this work, among the very few attempts to provide information in real conditions, paves the way to the possible homologation of EO formulas. Indeed, besides the proof of their efficacy provided here, EO encapsulation within emulsions appeared also as favorable formulation to improve the bioavailability of these volatile compounds, still keeping their properties, allowing the use of reduced concentrations. This, together with emulsion blending to exploit different EO modes of action, makes EO emulsions fulfilling another key criterion for new strategies of plant protection related to formulation costs.

Abbreviations

CIN	Cinnamon bark
CLO	Clove bud
EO	Essential oil
FDA	Food and Drug Administration
FIC	Fractional inhibitory concentration
FICI	Fractional inhibitory concentration index
GRAS	Generally recognized as safe
HG	Hepes glucose
KB	King's broth
MBEC	Minimal biofilm eradication concentration
MIC	Minimal inhibitory concentration
MS	Murashige and Skoog
OD	Optical density at 600 nm
ORE	Oregano
Psa	*Pseudomonas syringae* Pv. *actinidiae*
QS	Quorum sensing
Rpm	Rounds per minute
T3SS	Type three secretion system
THY	Thyme
USA	United States of America

Supplementary Information

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Additional file 1.

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Author contributions

The authors confirm contribution to the paper as follows: EV, FSp, MBon, LT, SL conceived and designed the study DD, MT, SC, FSa, GS, MBov, MA performed the experiments and collected the data DD, MT, FSpi, EV, AP, SL analyzed and interpreted the results DD, EV and FSpi prepared the draft of the manuscript and FSpi substantially revised All authors reviewed the results and approved the final version of the manuscript.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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