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ORIGINAL ARTICLE

Combining cyclic lipopeptides and cinnamon extract enhance antifungal activity against *Fusarium oxysporum* strains pathogenic to banana and delay Fusarium wilt under greenhouse conditions

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Abstract

Fusarium wilt of banana (FWB) caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is a widely distributed disease that generates devastating losses in banana production. *Foc* belongs to the *Fusarium oxysporum* species complex (FOSC) which includes several evolutionary lineages. Nine of them are pathogenic to banana such as *F. phialophorum*, *F. grosmichelli*, *F. duoseptatum* and the most aggressive *F. odoratissimum* tropical race 4 (TR4). No control method has been successfully implemented to manage FWB, then enhancing the potential of management approaches can avoid or delay disease epidemics and reduce disease severity. Here we determined the antifungal effect of different plant-based extracts against *Foc* in vitro, and whether the combination of cinnamon (*Cinnamomum zeylanicum)* extract and *Bacillus tequilensis* EA-CB0015 cyclic lipopeptides had an additive effect against different *Foc* lineages in vitro and against FWB in banana plants in greenhouse. We found, from 17 plant-based natural extracts, that cinnamon was highly active against *Foc* strain IB (race 1). Furthermore, cinnamon and cyclic lipopeptides inhibited different strains of various evolutionary lineages of *Foc* belonging to race 1 and TR4, and their combination increased in 1.4-fold the effect of the single extracts in vitro. Our results showed that soil concentration of *F. odoratissimum* TR4-II5 decreased by 1000-fold when treated with the combination of 488 mg L^{-1} cinnamon and 128 mg L^{-1} lipopeptides in a soil microcosm system after 5 days of incubation, followed by a partial population recovery after 21 days. In greenhouse experiments, the combination reduced external but not internal FWB symptoms, and cinnamon extract had a significant impact on internal plant symptoms. Taken together, the effect of cyclic lipopeptides with cinnamon extract on *Foc* supports their function towards delaying the effect of disease progression and suggests that the combination enhances the effect of the single extracts.

Keywords Lipopeptides · Cinnamon · Plant-based extracts · Soil health · Fusarium wilt of banana · Antifungal activity

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Introduction

Agricultural production is threatened by the dissemination of fungal pathogens that reduce yields of many crops worldwide (Fones et al. 2020). Bananas (*Musa* spp.) are produced mainly in tropical regions, generate around 22 million tons of fruit which are highly relevant as dietary basis of population in producing countries (FAO 2022). For several decades, bananas have been affected and devastated by Fusarium wilt of banana (FWB) (Dita et al. 2018). The disease is caused by the soil-borne *Fusarium oxysporum* f. sp. *cubense (Foc)*, which colonizes the vascular system of the banana plant, causing wilting, chlorosis of leaves, and finally the death of the infected plant (Guo et al. 2015; Li et al. 2017). *Foc* belongs to

the *Fusarium oxysporum* species complex (FOSC), which comprised distinct evolutionary lineages that cause vascular wilt diseases in economically important crops (Gordon 2017). Among them, nine lineages are grouped under the name of *Foc* because of their ability to induce wilt symptoms on bananas. They include *F. odoratissimum* (lineage 1) pathogen of Cavendish and Gros Michel bananas; *F. phialophorum* (lineage 3), *F. grosmichelii* (lineage 4) and *F. duoseptatum* (lineage 5), both pathogens of Gros Michel bananas (Maryani et al. 2019). Furthermore, the pathogen is grouped into races based on their ability to infect different banana cultivars (Waite and Stover 1960). Race 1 infects Gros Michel, whereas Cavendish bananas are resistant. Both Gros Michel and Cavendish cultivars are affected by tropical race 4 (TR4), which is the prime threat to banana cultivation (Dita et al. 2018).

Several management strategies have been implemented or tested against FWB including exclusion, eradication, resistant cultivars, crop rotation, chemical fungicides, organic amendments, and biological control agents (BCAs) (Dita et al. 2018; Siamak and Zheng 2018; Ismaila et al. 2022; Prigigallo et al. 2022). Nevertheless, considering the epidemiology of FWB and the perennial nature of monoculture banana plantations, it is evident that the management of the disease is not simple (Dita et al. 2018). Management practices oriented to soil health and suppressiveness could contribute to avoid or delay disease epidemics and disease severity. BCAs such as *Trichoderma* spp., *Pseudomonas* spp., *Bacillus* spp., and *Streptomyces* spp. have shown promising results to control FWB (Belgrove et al. 2011; Thangavelu and Gopi 2015; Bubici et al. 2019; Sun et al. 2021; Yun et al. 2022), nevertheless their effect has been centered in the application of microbial cells and less on using their active metabolites, many of which have antibiosis effect against phytopathogens (Köhl et al. 2019). *Bacillus* species from the Subtilis clade namely, *Bacillus subtilis, B. tequilensis, B. amyloliquefaciens and B. velezensis* (Patel and Gupta 2020), produce secondary metabolites including polyketides (PKs), ribosomally synthesized compounds, terpenes, siderophores and non-ribosomal peptides (NRPs) (Caulier et al. 2019; Cuellar-Gaviria et al. 2023). Within NRPs, cyclic lipopeptides such as the surfactin, iturin, and fengycin families have shown a broad antimicrobial activity against many important phytopathogens (Caulier et al. 2019) including *Fusarium* spp. (Arroyave-Toro et al. 2017; Cao et al. 2018; Mihalache et al. 2018; Adeniji et al. 2019; Kang et al. 2020; Moreno-Velandia et al. 2021; Fatima et al. 2023). Specifically, *B. tequilensis* EA-CB0015 have been shown to produce surfactin, fengycin and iturin lipopeptides, from which the purified mixture of the three families or the single lipopeptides fengycin and iturin have shown antifungal effect against different fungal pathogens (Mosquera et al. 2014; Arroyave-Toro et al. 2017; Cuellar-Gaviria et al. 2021). Nevertheless, significantly less is known about their effect on *Foc* and on strategies that could enhance their biological control activity.

Equally significant are plant-based natural products which are composed of a range of active metabolites that have insecticidal, virucidal, fungicidal, bactericidal, and antiparasitic properties (Hou et al. 2022; Khursheed et al. 2022). Antifungal activity has been shown for tea oil (Yue et al. 2020; Perumal et al. 2021), clove (Sharma et al. 2017), mint and thyme (Soleimani et al. 2022), eucalypt (Bhuyan et al. 2017), cinnamon (Velluti et al. 2004; Xing et al. 2014b; Lee et al. 2020) among others. Particularly, cinnamon (*Cinnamomum zeylanicum*)-based oil have been shown to cause growth inhibition and morphological alterations on *Fusarium proliferatum* (Velluti et al. 2004), *Fusarium verticilloides* (Xing et al. 2014b), *Raffaelea quercus -mongolicae* and *Rhizoctonia solani* (Lee et al. 2020), and the containing compounds eugenol, cinnamaldehyde and trans-cinnamaldehyde have shown strong antifungal activity against *F. oxysporum* (Xie et al. 2017; Marei and Abdelgaleil 2018) and *F. solani* (Pan et al. 2023).

Enhancing biological control efficacy of microbial and plant-based biopesticides through a combinatory approach is still limited and have huge potential (Arrebola et al. 2010; Zamani-Zadeh et al. 2014; Basaid et al. 2021; Dimkić et al. 2022). To our knowledge, few studies have been conducted to explore the effect of a combinatory approach of plant-based extracts and *Bacillus* lipopeptides against microbial pathogens (Olfa et al. 2015; Sudarmono et al. 2019; Bibi et al. 2021), and none against *Foc*. As plant-based extracts and cyclic lipopeptides may exert different modes of action against fungal pathogens, we tested the hypothesis that a combinatory approach of highly active plant-based extracts and bacterial cyclic lipopeptides have an additive antifungal effect against *Foc* that reduce the disease severity of FWB disease in banana plants. To gain insights into the combinatory approach hypothesis, we used in vitro assays, microcosm and greenhouse experiments using strains of different evolutionary lineages. Overall, this study reveals that combining cyclic lipopeptides and cinnamon extract enhanced antifungal activity against *Fusarium oxysporum* strains pathogenic to banana and delayed progression of *Fusarium* wilt under greenhouse conditions.

Material and methods

Microorganisms and culture conditions

Bacillus tequielensis EA-CB0015 (NCBI reference sequence NZ_CP048852.1), previously isolated from the phyllosphere of cv. Grand Naine in Urabá, Antioquia, Colombia (Ceballos et al. 2012; Villegas-Escobar et al. 2013) was activated from

frozen cultures on 50% TSA (Tripticase Soy Agar, Merck) for 48 h at 30 °C before any experimental use. Strains of *Foc* IB (GenBank OR698889 and OR698890), *F. phialophorum* CR1.1A (CR1.1A), *F. odoratissimum* II5 (TR4-II5), and *F. odoratissimum* II5:GFP (TR4-II5:GFP) from the Laboratory of Phytopathology – Wageningen University preserved in filter papers, were activated on PDA at 25 °C for 7 days. Strains CR1.1A and IB group into race 1 affect Gros Michel cultivar but not Cavendish, whereas strain TR4-II5 group in TR4 affects both cultivars. For microcosm and plant assays, spores of the isolates TR4-II5:GFP and TR4-II5 were resuspended in sterile water and concentrations were adjusted to $(1.5 \pm 0.5) \times 10^6$ and 2.0×10^6 spores mL^{-1}, the inoculum comprised various structures of micro and macroconidia.

Bteq EA-CB0015 lipopeptides and plant extracts

Production, extraction and purification of lipopeptides from *Bteq* EA-CB0015 were performed as previously described (Villegas-Escobar et al. 2013; Mosquera et al. 2014). The mixture comprising iturin, fengycin, and surfactin isoforms was stored at 4 ºC until use.

A total of 17 plant-based natural extracts were used in this study (Table 1). Essential oil extracts from chamomile, lavender, eucalyptus, rosemary, tea tree, cinnamon, mint, and garlic, were obtained from FUNAT company (Medellín, Colombia). Citronella extract was obtained from Green company (Bogotá, Colombia), while the remaining compounds were obtained by hydro-distillation or supercritical extraction following previously described methodologies (Ghoreishi and Bataghva 2011; Roohinejad et al. 2017). For instance, cinnamon extract was obtained through hydro-distillation by immersing cinnamon sticks (60 g) in a 350 mL ethanol (96%) bath that was heated to boiling point. The decanted extract was concentrated to a solid residue using a rotary evaporator, resulting in 0.051 g cinnamon extract g^{-1} cinnamon sticks. When needed, extracts were diluted in methanol, and stored at 4 ºC.

In vitro assays

Well-diffusion assay

The well-diffusion assay was used to determine the effect of different natural extracts on *Foc* IB, and to determine the effect of lipopeptides and cinnamon extract on the mycelial growth of different *Fusarium* strains (IB, CR1.1A and TR4-II5). For each treatment, 20 µL of the tested compound was added to two punched wells placed at opposite sides of the Petri dish and a 5 mm fungal plug was placed in the center. Water or methanol was used as negative control. Plates were incubated at 26 °C in the dark and the radio (mm) of the fungal colony was recorded with a caliper after 7 days of growth. The percent inhibition for each treatment was calculated considering the fungal growth in the negative control as 100%. The effect of the combination of lipopeptides and the cinnamon extract was evaluated using different treatments: 1) lipopeptides (50 mg L^{-1}, 20 µL), 2) cinnamon (50 mg L^{-1}, 20 µL), 3) mixture of lipopeptides (100 mg L^{-1}, 10 µL) and cinnamon (100 mg L^{-1}, 10 µL). All experiments were performed in triplicates in a complete randomized design.

Table 1 Plant extracts used for bioprospecting assay

Natural source	Specie	Source	Method
Chamomile	*Chamaemelum nobile*	Commercial	Not determined
Cinnamon	*Cinnamomum zeylanicum*		
Citronella	*Cymbopogon winterianu*		
Eucalyptus	*Eucalyptus globulus*		
Garlic	*Allium sativum*		
Lavender	*Lavandula officinallis*		
Mint	*Mentha piperita*		
Rosemary	*Salvia rosmarinus*		
Tea tree	*Melaleuca alternifolia*		
Bejuco	*Sarcostemma glaucum*	This study	Hydro-distillation extraction
Cinnamon	*Cinnamomum zeylanicum*		
Eucalyptus	*Eucalyptus globulus*		
Mint	*Mentha piperita*		
Tea tree	*Melaleuca alternifolia*		
Cinnamon	*Cinnamomum zeylanicum*		Supercritical fluid extraction
Tea tree	*Melaleuca alternifolia*		
Mint	*Mentha piperita*		

Agar dilution assay

The effect of the combination of lipopeptides and the cinnamon extract was evaluated by the agar dilution method described previously (Wiegand et al. 2008). A mixture of lipopeptides and cinnamon extract was incorporated in different proportions into PDA medium, where the volume of the mixture corresponded to 5.0% of the total medium volume. Then, 20 µL of a *Foc* IB spore suspension (1.5×10^5 spores mL^{-1}) composed of micro and macroconidia, were applied in four punched wells. The following proportions of lipopeptides and cinnamon extracts were tested (100:0, 80:20, 60:40, 40:60, 20:80, 0:100), where the 100% values corresponded to 122 mg L^{-1} for cinnamon extract and 64 mg L^{-1} for lipopeptides. Plates were incubated at 30 °C in the dark and fungal growth was measured at 4-day post inoculation. All experiments were performed in triplicate in a complete randomized design.

To evaluate the effect of combinations two approaches were applied. The Highest Single Agent approach reflects the fact that the resulting effect of a combination (E_{AB}) is greater than the effects produced by each component separately (E_A and E_B) (Lehár et al. 2007). Then a combination index was calculated as $CI = max(E_A, E_B)/E_{AB}$ and the significance of a positive effect was determined by the *P-value* of the statistical test comparing the combination (E_{AB}) to the highest single agent ($\max(E_A, E_B)$) (Foucquier and Guedj 2015). On the other hand, the Bliss Independence model, based on the principle that compounds effects are outcomes of probabilistic processed and considers that components act independently without interfering with each other (Geary 2013), was determined calculating the combination index as $CI = (E_A + E_B - E_A E_B)/E_{AB}$ (Foucquier and Guedj 2015). Then if $CI < 1$, the combination (E_{AB}) was interpreted as a synergistic effect with respect to the single agents.

Soil microcosm experiment

The effect of the mixtures of lipopeptides and cinnamon extracts on the growth dynamics of TR4-II5:GFP into the soil substrate was evaluated in a microcosms model bioassay under a complete randomized design. Ninety (90) g of soil substrate (Swedish sphagnum peat, grinding clay granules, garden peat, beam structure, steamed compost, PG-Mix-15–10-20 (group of NPK: with 15% of nitrogen, 10% of phosphorus, and 20% of potassium)) in 450 mL pots was uniformly inoculated with *Fusarium* TR4-II5:GFP in order to obtain a final concentration of 1×10^6 spores g^{-1}, the inoculum comprised mixture of micro and macroconidia. After 30 min of incubation, a solution of 10 mL of methanol with the different treatments was mixed into the soil substrate. The treatments comprised lipopeptides (128 mg L^{-1} or 256 mg L^{-1}), cinnamon extract (244 ppm or 488 mg L^{-1}), mixture 1 (cinnamon at 244 mg $L^{-1}$and lipopeptides at 64 mg L^{-1}), and mixture 2 (cinnamon at 488 mg $L^{-1}$and lipopeptides at 128 mg L^{-1}), with methanol or water as negative controls and an absolute control without *Fusarium* inoculation was also included. In total four biological replicates (pots) per treatment were used, and all pots were incubated at 28 ± 2ºC, 16 h light, and 85% relativity humidity in a greenhouse compartment. Soil substrate moisture was maintained at field capacity.

Quantification of TR4-II5:GFP

To measure the growth dynamics of TR4-II5:GFP, 1 g of soil samples were taken at 0, 1, 2, 3, 5, 6, 14 and 21 days after inoculation. The TR4-II5 concentration was determined by counting of colony-forming units per gram of soil (CFU g^{-1}). Serial dilutions and plating in PDA modified medium (streptomycin sulfate (0.3 g L^{-1}), tetracycline (10 mg mL^{-1}), and hygromycin (10 mg mL^{-1})) was performed and only colonies expressing GFP were count under UV lamp at 254 nm. For each pot, 3 technical replicates of 1 g each were used and then averaged. The repeated measurements of CFU g^{-1} were merged into a single response variable (area under the curve - AUC) for each replicate, and then the AUC was used for analysis of variance. AUC was calculated through GraphPad Prism 9.3.0 software by the trapezoidal method.

We also determined the TR4-II5:GFP concentrations by q-PCR. DNA was extracted from the soil using the PowerLyzerTM PowerSoil DNA extraction kit. A standard curve was determined by using the primers qPCR_TR4_F/qPCR_TR4_R (5′CTCTATATCACATAGTAGAAAAACAAGTAAACGAGC/5′CATATATGGGACCTTTATGAATGCGAGAATGGGGAT) with a melting peak of 82ºC. Based on Ct values against the amount of genomic DNA a standard curve was created with two-fold serial dilutions of the gDNA of TR4 in triplicate real-time reactions. The thermal cycling conditions consisted of an initial denaturation for 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, annealing at 60 °C for 15 s, extension at 72 °C for 30 s. After the q-PCR, the products were analyzed by melting curves (65 °C to 99 °C) through CFX manager software to verify their specificity.

Primer Express (version 3.0; Applied Biosystems, Foster City, CA) was used to design the specific q-PCR primers. The q-PCR was performed on a CFX96TM Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA). SensiMixTM SYBR® Hi-ROX Kit (Novoprotein, Shanghai, China) was used to detect the concentration of TR4 in the soil treatments and data analysis considered the Ct value to interpolate it in the standard curve. For each treatment, at least four biological repetitions (four pots) with three technical replicates were conducted.

Greenhouse experiment

The effect of lipopeptides and cinnamon extract mixtures on banana plants inoculated with TR4-II5 was evaluated under greenhouse conditions (García-Bastidas et al. 2019) following a complete randomized block design. Cavendish bananas were planted in 1 kg of soil in 1000 mL pots (one plant/pot). The plants were inoculated by pouring 200 mL of TR4-II5 inoculum (1×10^6 spores/mL) directly to the soil of potted banana plants and then the roots of each plant were mechanically wounded twice around the corm. After 24 h of incubation, 10 ml of the different treatments were applied into the soil substrate as a drench, where the transplanted plants were already sown. The treatments were comprised of T1 lipopeptides (128 mg L^{-1}), mixture T2 (cinnamon at 244 mg $L^{-1}$and lipopeptides at 128 mg L^{-1}), mixture T3 (cinnamon at 122 mg $L^{-1}$and lipopeptides at 64 mg L^{-1}), and T4 cinnamon extract (244 mg L^{-1}). Two negative controls were used, one with water (control 1) and the other with methanol (control 2). An absolute control with no TR4-II5 application was included. In total 5 biological replicates per treatment were used and all plants were placed in an environmentally controlled greenhouse compartment (28 ± 2 ºC, 16 h light, and approximately 85% relativity humidity) for 42 days.

Disease severity was determined 42 days after inoculation by calculating the disease index with the following equation (Cao et al. 2011).

$$DI(\%) = \left[\Sigma\left(n_i \times v_i\right) \div (V \times N)\right] \times 100$$

where n_i indicates the number of plants within a disease scale; v_i= disease scale; V = highest value of the disease scale (5) and N = the number of plants evaluated. The disease scale corresponds to the following values: 0 = no symptoms, 1 = one to two leaves with yellowing, 2 = three leaves with yellowing, 3 = four leaves with yellowing, 4 = five or more leaves with yellowing and 6 = withered plant. Additionally, 42 days after pathogen inoculation, internal disease symptoms were recorded by cutting the corm of the plant. Specifically, the percentage of necrosis was calculated by comparing the necrotic area with the total corm area with ImageJ software (Hériché et al. 2022).

Data analysis

Analysis of variance (ANOVA) was used to analyze each experiment in GraphPad Prism 9.3.0 (GraphPad Software Inc, California, USA, using 95% confidence limits). The assumptions of normality (Shapiro-Wilks test), homoscedasticity (Levenne's test), and independence (graphic residues vs. run order) were tested and confirmed. Tukey's multiple range test was applied to determine significant differences among treatments in all the experiments.

Results

Cinnamon extract has a notable inhibitory effect against *Foc*

To explore the antifungal effect of 17 natural extracts against *Foc* IB an in vitro bioprospecting assay based in the well-diffusion assay was performed. In general, cinnamon (commercial, supercritical, hydro-distillated source), mint (commercial source), citronella (commercial source) and tea (commercial source) extracts significantly inhibit the fungal growth when compared to non-treated control (Table 2, supplementary material Fig. S1), while chamomile (commercial source), garlic (commercial source), bejuco (HD), mint (HD), tea (HD) did not. Consequently, cinnamon extract obtained through hydro-distillation, was selected for further assays as having the highest activity (commercial 71.22%, supercritical 61.3%, hydro-distillation 37.7%).

Cinnamon extract and lipopeptides inhibit *Fusarium* spp. in a strain-dependent manner and have a synergistic antifungal effect

To determine whether the cinnamon extract and lipopeptides have an inhibitory effect on the TR4-II5, *Foc* IB and CR1.1A strains, a well-diffusion assay was performed (Fig. 1A). In general, all single (cinnamon 50 mg L^{-1}, lipopeptides 50 mg L^{-1}) and combined (cinnamon 50 mg L^{-1} + lipopeptides 50 mg L^{-1}) extracts affected the fungal growth with inhibition percentages above 36%, but this inhibition was strain dependent (Fig. 1A). Cinnamon extract displayed a higher inhibition effect against TR4-II5 (44.5%), lipopeptides were more active against *Foc* IB (55.9%) and CR1.1A (54.1%), while the combination was more active against *Foc* IB (60.1%) and TR4-II5 (54.2%), suggesting that *Fusarium* spp. strains have different sensitivities against active compounds in the cinnamon extract and the lipopeptides. Additionally, the combination of the two extracts slightly increased the inhibition compared to the individual extracts for strains TR4-II5 and *Foc* IB (Fig. 1A), suggesting a synergistic effect.

To test if the combination of cinnamon and lipopeptides had a positive effect (synergy), we used two methods (highest single agent, bliss independence) through the agar dilution assay against *Foc* IB (Fig. 1B, C, Fig. S2). With both methods the combination of cinnamon and lipopeptides in a proportion 80:20 had a CI less than 1 indicating a synergy effect. Moreover, the combination had a significant effect showing a 1.4-fold increase compared to the highest single

Table 2 Antifungal activity of natural extracts against *Foc* IB growth in solid culture medium. The commercial extracts were tested at the concentration stated by the manufactured: chamomile extract and garlic extract at 0.2 g/mL; the oils cinnamon, citronella, eucalyptus, lavender, mint and tea tree were 100% pure oil. Extracts obtained by hydro-distillation and supercritical fluid extraction were tested at 0.2 g/mL

Source	Natural product	Radial growth (cm)	% inhibition
Commercial	Chamomile	4.25 ± 0.11 [a]	-5.46
	Cinnamon	1.16 ± _0.06 g	71.22
	Citronella	1.93 ± _0.19 [e]	52.11
	Eucalyptus	3.60 ± _0.14[b]	10.67
	Garlic	4.25 ± 0.11 [a]	-5.46
	Lavender	3.45 ± _0.35 [b]	14.39
	Mint	1.93 ± _0.25[e]	52.11
	Rosemary	3.50 ± 0.12 [b]	13.15
	Tea	1.96 ± _0.06 [e]	51.36
This study – Hydro-distillation	Bejuco	4.20 ± _0.16 [a]	-4.22
	Cinnamon	2.51 ± _0.08 [d]	37.72
	Eucalyptus	2.97 ± _0.30 [c]	26.30
	Mint	4.11 ± _0.03 [a]	-1.99
	Tea	4.06 ± _0.06 [a]	-0.74
This study—Supercritical Fluid	Cinnamon	1.56 ± _0.21 [f]	61.29
	Mint	2.96 ± _0.12 [c]	26.55
	Tea	3.36 ± _0.15 [b]	16.63
	Negative control	4.03 ± _0.17 [a]	NA

Means with the different letter differ statistically (*p-value* = 0.0001) by Tukey multiple range tests. Standard error of the mean is presented by ± (n = 3)

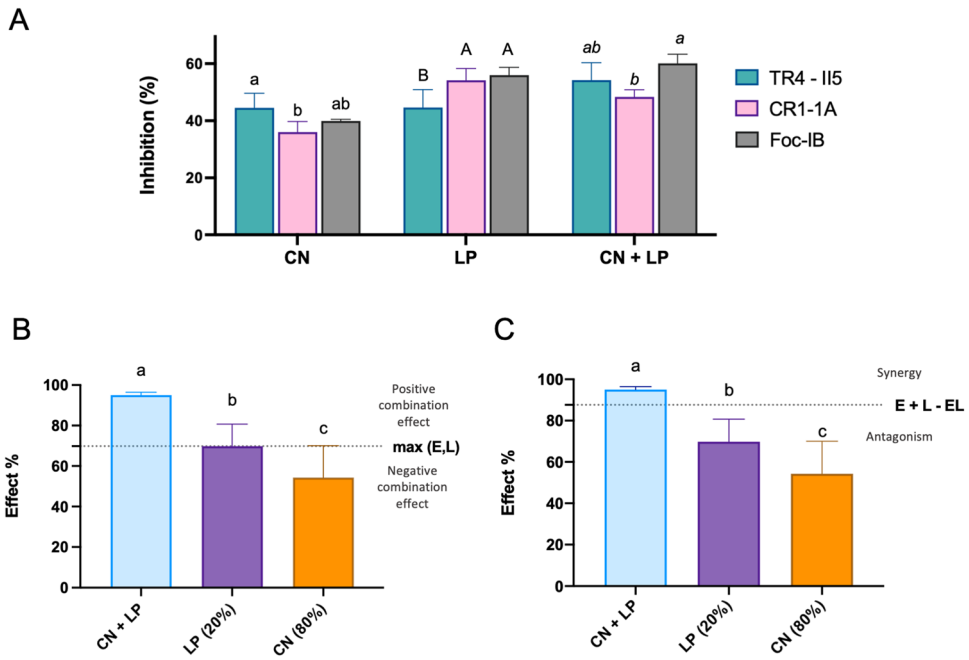

Fig. 1 Antifungal activity of cinnamon extract (CN) and lipopeptides (LP) in solid culture medium. A. Activity of cinnamon, lipopeptides and the mixture on the three fungal strains (TR4-II5, CR1-1A, Foc-IB). B. Highest single agent approach showing the positive combination of cinnamon extract and *Bteq* EA-CB0015 lipopeptides (E = Cinnamon extract, L = Lipopeptides) on *Foc*-IB. C. Bliss independence approach showing the synergy of the combination of cinnamon extract and *Bteq* EA-CB0015 lipopeptides on *Foc*-IB. Different letters above each bar indicate significant differences according to the Tukey's multiple comparison tests *p-value* = 0.045 (Fig. 1A – lowercase), *p-value* = 0.028 (Fig. 1A – uppercase), *p-value* = 0.005 (Fig. 1A – cursive lowercase), *p-value* < 0.0001 (Fig. 1B, Fig. 1C). Standard deviation is presented by vertical bars (n = 3)

agent (lipopeptides) (Fig. 1B, 1C). The other proportions of cinnamon and lipopeptides tested 60:40, 40:60 show a positive effect of 1.04-fold and 1.07-fold increase of the combination compared to the highest effect observed for a single agent (lipopeptides), meanwhile the proportion 20:80 did not exhibit positive effect (supplementary material, Fig. S2B).

Cinnamon extract and lipopeptides reduce TR4-II5 concentration in soil

To determine whether cinnamon extract and lipopeptides can reduce TR4-II5-GFP concentration on soil, a microcosm assay was performed. In general, all single and combined extracts reduced the concentration (CFU g^{-1}) of TR4-II5-GFP during the experiment (supplementary material Fig. S3A). After 5 days of incubation, viable cells of *Fusarium* TR4-II5-GFP were reduced by 35 to 60-fold by lipopeptides at 128 mg L^{-1} and 256 mg L^{-1}, 27 to 288-fold by cinnamon at 244 mg $L^{-1}$and 488 mg L^{-1}, and by 229 to 1000-fold the combination T1 (64 mg L^{-1} lipopeptides, 244 mg L^{-1} cinnamon) and T2 (128 mg L^{-1} lipopeptides, 488 mg L^{-1} cinnamon) respectively (supplementary material Fig. S3A, Fig. 2A, Table S1). Likewise, qPCR results showed a similar pattern, all treatments reduced the concentration of TR4-II5-GFP by an average of 2.8-fold after 5 days of incubation (Fig. 2B). Interestingly after 21 days of incubation, an increase in CFU g^{-1} was detected for all treatments reducing viable cells of TR4-II5-GFP in average by 18 fold, but despite this change, there was still significant differences between treatments and the negative controls (supplementary material Fig. S3A, Fig. 2A, Table S1). These results show that the inhibitory effect increased in the most concentrated treatments, showing a dose-dependent effect of the compounds (lipopeptides, cinnamon extract and their combination) against TR4-II5.

To determine if the combined extracts had a differential effect, the repeated measurements of CFU g^{-1} were collapsed into a single response variable (area under the curve) (Fig. 2C). In general, all treatments induced a significant reduction in AUC compared to the non-treated control, but only the combined treatment T2 had a greater effect than the single treatments. In general, the most concentrated treatments (T2) had a greater reduction in the concentration of CFU g^{-1} compared to the least concentrated treatments (T1), suggesting a dose dependent effect related to the fungal inhibition treated with lipopeptides, cinnamon or both.

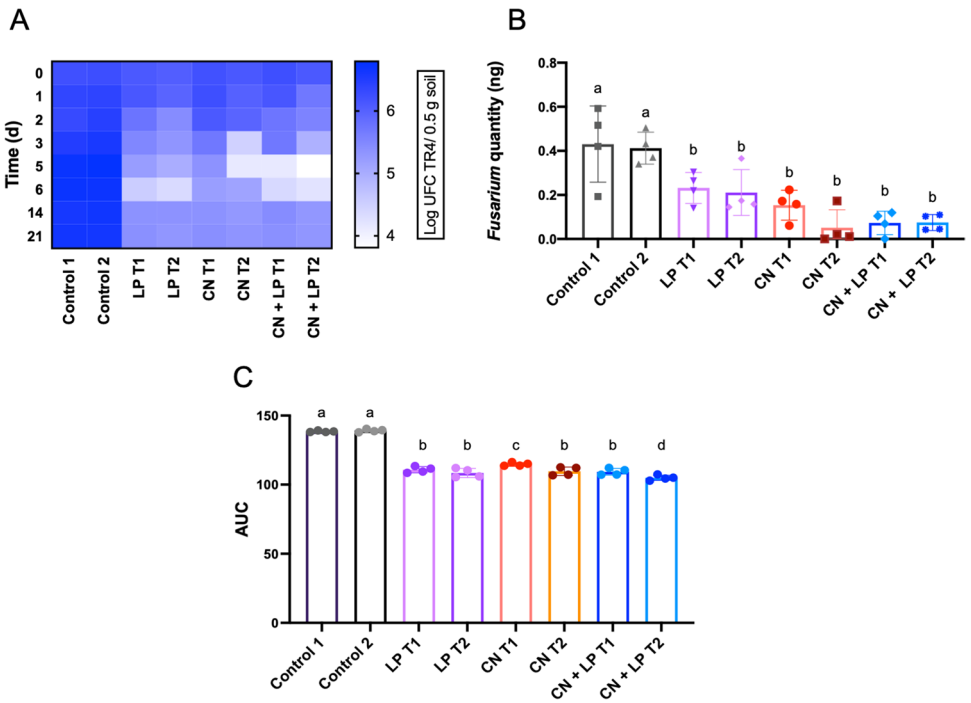

Fig. 2 Effect of lipopeptides (LP) and cinnamon extract (CN) on the growth dynamic of *Fusarium* sp. TR4-II5 under microcosms soil conditions. A. Heatmap visualization of the dynamic of TR4-II5 CFU g^{-1} over time. B. Concentration of TR4-II5 on microcosm soils after day 5 of incubation based on standard curve (supplementary material Fig. S3) of the TR4-II5 qPCR. C. Area under curve- AUC (CFU g^{-1}*days) of the dynamics of CFU g^{-1} through time (supplementary material Fig. S3). Different lowercase symbols represented a significant difference according to the Tukey's multiple comparison test *p-value* < 0.0001 (Fig. 2B), v*p-value* = 0.0006 (Fig. 2C). Standard deviation is presented by vertical bars ($n = 4$). Treatments: Control 1: H_2O, Control 2: MeOH. Lipopeptides T1:128 mg L^{-1} and T2: 256 mg L^{-1}, Cinnamon extract T1:244- mg L^{-1} and T2: 488 mg L^{-1}, Mixture T1: Cinnamon (244 mg L^{-1}) and Lipopeptides (64 mg L^{-1}), and T2: Cinnamon (488 mg L^{-1}) and Lipopeptides (128 mg $L.^{-1}$)

Cinnamon extract reduces *Fusarium* wilt of banana under greenhouse conditions

To investigate the potential reduction of FWB by cinnamon extract, lipopeptides, and their combination a greenhouse trial was performed and scored 42 days after inoculation (Fig. 3). All treatments reduced the external symptoms of the disease compared to the negative control (Fig. 3A), reducing them from 68.9% (control 1) to 48.9% (LP), 36.2% (CN), 44.7% (CN + LP1), and 48.3% (CN + LP2). Particularly, the CN and CN + LP1 reduced plant wilting and chlorosis compared to the control group (supplementary material Fig. S4). In contrast, internal symptoms were only significantly reduced by 1.9 fold in the CN compared both controls (Fig. 3B).

Discussion

Biopesticides including BCAs and plant-based natural products are a potential and important strategy for crop protection. The application of active ingredients (metabolites) and their combination face key challenges for future implementation including inefficient production and recovery methods, production costs, persistence, heavy regulatory process of approval and limited efficacy compared to synthetic fungicides (Pavela and Benelli 2016; Basaid et al. 2021). Thus, research oriented towards discovering effective metabolites with possible additive and synergistic effects should be prioritize. The current dissemination of *Fusarium* TR4 around the world is worrisome and causes significant yield reductions, hence alternative disease control strategies are required (Dita et al. 2018; Kema et al. 2021). Therefore, in evaluating the combinatory effect of lipopeptides and cinnamon extract, we uncovered the basis of a synergistic or additive antifungal activity against *Foc* that could potentiate management practices for FWB. To obtain this synergistic effect, a bioprospecting assay was conducted with 17 plant-based natural extracts and *Bteq* EA-CB0015 lipopeptides in vitro against *Foc*. Then, the combined effect of the most promising extract (cinnamon) and lipopeptides was evaluated under in vitro conditions, soil microcosm and in banana plants.

The bioprospecting assay unveils the superior inhibitory effect of cinnamon extract. This plant extract has already been reported for its antifungal activity on *F. oxysporum*, *F. verticillioides*, *F. proliferatum* and *F. graminearum*, with irreversible ultrastructural alterations, inhibition of

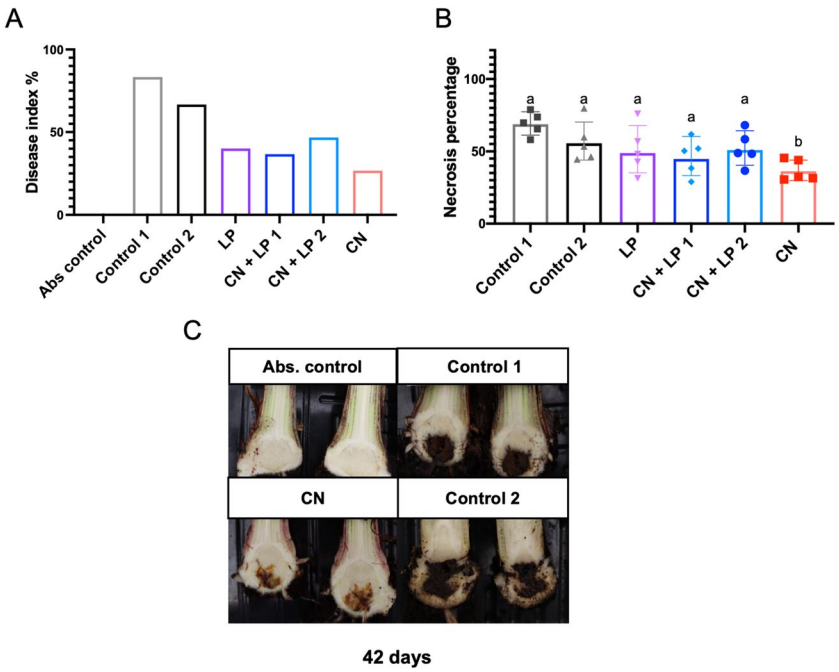

Fig. 3 Effect of lipopeptides and cinnamon extracts on FWB symptoms caused by TR4-II5 under greenhouse conditions at 42 dpi. A. Disease index (%) of external symptoms. B. Quantification (Image J) of internal necrosis of the corm of Cavendish plants due to FWB in the various treatments compared to the controls. C. Necrosis in sectioned Cavendish corms after the CN treatment compared to the controls. Different lowercase symbols represented significant difference according to the Tukey's multiple comparison test (*p—value* = 0.01). Standard deviation is presented by vertical bars ($n = 5$). Treatments: Control 1: H_2O, Control 2: MeOH, LP (128 mg L^{-1}lipopeptides), CN + LP 1 (244 mg L^{-1} cinnamon – 128 mg L^{-1} lipopeptides), CN + LP 1 (122 mg L^{-1} cinnamon – 64 mg L^{-1} lipopeptides), CN (244 mg $L.^{-1}$ cinnamon)

enzymatic reactions of cell wall synthesis, interference with lipid membranes of fungal cells, increasing of cation permeability in membranes and effects on secondary metabolites production (Velluti et al. 2004; Xing et al. 2014a, 2014b; Guo et al. 2020). Our results support these observations regarding the antifungal activity of cinnamon extract and reports for the first-time activity against *Foc* (race 1 and TR4). The inhibition of mycelial growth was > 36% for all strains, however we obtained a higher effect of cinnamon extract on TR4-II5 than on CR1.1A and *Foc* IB, possibly due to the genetic differences between these species that were previously considered as lineages of *F. oxysporum* f. sp. *cubense*. Race 1 (CR1.1A and *Foc* IB) can affect Gros Michel cultivar but not Cavendish, whereas TR4 affects both cultivars (Maryani et al. 2019).

Similarly, *Bteq* EA-CB0015 lipopeptides inhibited all tested strains with inhibitions > 44%, but the effects were strain dependent. Furthermore, the combination of both extracts in an 80:20 proportion (Cinnamon-Lipopeptides), showed a synergistic effect on *Foc* IB in vitro, following the bliss independence and highest single agent approaches (Goldoni and Johansson 2007). Synergistic effects have not been proven with cinnamon extract and lipopeptides against *Fusarium* spp., however, the combination approach has been tested for various human pathogens, showing drug synergy between antibiotics and lipopeptides such as colistin, bacillomycin, and surfactin against *Candida* spp., *Acinetobacter* spp., *Pseudomonas* spp. (Olfa et al. 2015; Sudarmono et al. 2019; Bibi et al. 2021). Therefore, understanding the nature of the synergistic activity between lipopeptides and cinnamon will help in the finetuning of their application for disease treatment.

The combined effect of cinnamon and lipopeptides was tested in a soil microcosm experiment since FWB is a soilborne disease. All single and combined treatments reduced TR4-II5 concentration in the soil after 21 days when applied once, but concentrated mixtures had the highest antifungal effect, suggesting a dose-dependent mode of action. Although microcosm and in vitro assays are interesting approximations of the fungal response to treatments, they do not mimic the in vivo conditions, and ignore the interactions with the microbiome inside the plant or in the soil (Fu et al. 2017; Yang et al. 2022).

Therefore, our study also explored the response in plant assays, showing a reduction of internal FWB symptoms in infected Cavendish plants after a cinnamon extract treatment (36%) at 42 dpi, but also on external symptoms by the combinatory approach. The effect of the cinnamon and lipopeptides combination on the biomass of *Foc* was found to be partial and concentrated at the beginning of the microcosm experiment, with a partial recovery afterwards, tendency that could be the cause of the delayed progression of the disease, as showed in the evaluation of the external symptoms. These results are comparable with a disease index reduction of 20% after the treatment with *Streptomyces* spp., at 49 dpi (Li et al. 2021), and the reduction of 10% in FWB incidence by *B. licheniformis* CSR-D4 and its lipopeptides (Yadav et al. 2021). Although the secondary metabolites of bacteria and natural extracts represent a good alternative in the in vitro reduction of TR4, the reduction of FWB is lower, therefore, such treatments should be considered as possible disease mitigation rather than eradication methods. Compared to the in vitro and microcosm assays, this experiment involves the fungus versus the plant – soil – microbiome complex and shows the effect of cinnamon extract for FWB reduction. However, more trials are required to obtain a comprehensive overview of the efficacy of these mixtures at various concentrations for FWB reduction. Furthermore, the application of pure lipopeptides with cinnamon extract as evaluated in this study, may not be cost effective, but opens new research questions to study the effect of the application of biopesticides based on lipopeptide producer strains (e.g. *Bacillus tequilensis*) and cinnamon extract. Also, incorporating biopesticides based on the combination of bacterial strains and plant-based extracts to the soil, also opens new research avenues regarding their effect on soil microbial ecology.

In conclusion, this study explores bioprospecting with natural products against *Fusarium* spp. The results showed the synergistic effects of lipopeptides and cinnamon in vitro against *Foc* IB and TR4-II5, and the decrease of TR4-II5 concentration in soil. We also showed efficacy of cinnamon extracts in delaying the progression of disease symptoms in plant assays. This study provides an initial approximation of these bioactive antifungal compounds as adjuvants in the biological control of TR4.

Authorship contributions

Conceptualization (LAG, VVE, JMRM); Data curation (JMRM); Formal analysis (JMRM); Funding acquisition (LAG, VVE, SZH); Investigation (JMRM); Methodology (LAG, VVE, JMRM, SZH); Project administration (LAG, VVE); Resources (LAG, VVE, CAG, GHJK, SZH); Supervision (LAG, VVE, CAG, GHJK); Writing – original draft (JMRM); Writing – review & editing (LAG, VVE, JMRM, CAG, GHJK, SZH). All the authors have read the paper and have agreed to be co-authors.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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