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Lipopeptides from *Bacillus tequilensis* EA-CB0015 and cinnamon extract decrease the bioenergetic response of *Fusarium oxysporum* f. sp. *cubense*

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Abstract Lipopeptides (LPs) from *B. tequilensis* EA-CB0015 have antifungal activity against *Fusarium* species. Specifically, against *F. oxysporum* f. sp. *cubense* (*Foc*), the cause of *Fusarium* wilt of banana. Cinnamon (CN) extract is also known for its inhibitory activity against the *Fusarium* genus. The main goal of this research was to determine whether the effect of LPs and CN extract or their combination against a *Foc* isolate is related to an impairment of mitochondrial function. Our results show that biomass decreased by 74% ($p < 0.0001$) and 84% ($p < 0.0001$) when cultures were treated with 128 ppm LPs and 152.5 ppm CN extract, respectively. In parallel, we found a pronounced impairment of the bioenergetic response. That is, the routine oxygen consumption rate diminished by 55% ($p = 0.0148$) and 38% ($p < 0.0001$), respectively. Moreover, the ATP-linked respiratory rate decreased by 63% ($p = 0.0461$) and 44% ($p = 0.0005$), while the FCCP-simulated respiratory rate by 63% ($p = 0.0255$) and 45% ($p = 0.0002$). Therefore, our data suggest that the altered bioenergetic response observed in cultures of *Foc* treated with LPs or CN is mainly caused by an impairment of the activity of the respiratory complexes. On the other hand, biomass production was reduced by 80% ($p < 0.0001$) when cultures were treated with a mixture comprising only 10% LPs and 40% CN extract. Furthermore, ATP-linked and FCCP-stimulated respiratory rates decreased by 62% ($p = 0.0024$) and 68% ($p < 0.0001$), respectively under the same conditions. A potentially synergistic antifungal effect of cyclic LPs with a CN extract is suggested.

Highlights
- Cinnamon (CN) and cyclic lipopeptides (LP) inhibit growth of *Fusarium* spp.
- CN and LPs affect *F. oxysporum* f. sp. *cubense* (*Foc*) bioenergetic response.
- *Foc* bioenergetics reveals respiration is mostly associated to ATP synthesis.
- The combination of LPs and CN appears to synergistically inhibit *Fusarium* spp.

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Introduction

Fungal plant diseases are a significant threat to agricultural production. They cause substantial crop yield losses, which, in turn, has a negative impact on the economy and global food security (Sharma & Manhas, 2020). Such is the case for Fusarium Wilt of Banana, a widely distributed disease caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (Mon et al., 2021; Ploetz, 2015). An eradication method for the Fusarium Wilt of Banana is not currently known (Ismaila et al., 2022); however, biological control is a very promising approach to protect banana crops against *Foc* (Abram et al., 2021; Dimkić et al., 2022; Köhl et al., 2019). In this regard, microbial secondary metabolites have proven to produce direct antibiosis against fungal pathogens (Köhl et al., 2019). Moreover, the *Bacillus subtilis* complex, which comprises *Bacillus amyloliquefaciens, B. subtilis, B. velenzensis and B. tequilensis* species, is known for its antifungal properties, due to metabolites such as polyketides (PKs), siderophores and non-ribosomal peptides (NRPs) (Caulier et al., 2019; Labiadh et al., 2021). Specifically, cyclic lipopeptides (LPs), a class of metabolites that comprises isoforms of fengycin, iturin and surfactin families, isolated from *Bacillus tequilensis* EA-CB0015, are biomolecules found to have prominent activity against *Fusarium* spp. (Arroyave-Toro et al., 2017). Despite the fact that LPs represent a promising alternative in the control of phytopathogens such as *Fusarium* spp., it is necessary to improve their efficacy and cost of production to compete with agrochemical methods. In this respect, an encouraging strategy is to look for combinations of biological compounds with a high potential to cause synergistic antifungal effects. Nevertheless, this approach needs additional research efforts in order to answer questions such as the underlying molecular or cellular events that determine their antifungal effect.

Plant-based extracts are another interesting approach against phytopathogens. These are composed of a broad range of organic bioactive compounds, which are known to have insecticidal, fungicidal, bactericidal, or anti-parasitic properties (Gonçalves et al., 2021). The antifungal effect of plant-based extracts has been reported to involve hydrophobic interactions with the membrane of the host organism, alterations of the cellular structure and affections of metabolic pathways (An et al., 2019; Bi et al., 2021; Feng et al., 2019; Roohinejad et al., 2017). Specifically, cinnamon (*Cinnamomum zeylanicum*) extract is known for its antifungal effect on the *Fusarium* genus, which is associated with increased production of reactive oxygen species (ROS), morphological alterations, and growth inhibition (Lee et al., 2020; Shreaz et al., 2016; Velluti, 2003; Xing et al., 2014a, 2014b). However, there is still a gap in knowledge as to the exact cellular targets of those extracts.

Some studies suggest that alterations of the mitochondrial function may be one underlying factor of the antifungal effect of bacterial LPs (Cárdenas-Monroy et al., 2017; de Zoysa et al., 2018; Patkar et al., 2012). Furthermore, there is evidence that *Bacillus* LPs decrease ATPase activity in fungal cells (Patkar et al., 2012), and that iturin A generates alterations in energy metabolism in *Aspergillus carbonarus* (Jiang et al., 2020). In addition, evidence from biophysical studies indicates that changes in the lipid milieu of the plasma membrane are among the fundamental factors that mediate fungal growth inhibition by bacterial lipopeptides. For instance, an alteration of the thermotropic phase transition of multilamellar vesicles of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was correlated to the action of fengyin C (González-Jaramillo et al., 2017).

Furthermore, cinnamon (CN) extract has been reported as inhibitor of secondary metabolism on *Fusarium verticilloides* (Velluti, 2003; Xing et al., 2014a), and compounds of cinnamon extract such as trans-cinnamaldehyde, neral, and geranial are reported to produce ROS generation and cell membrane disruption on *Raffaelea quercus-mongolicae* and *Rhizoctonia solani* (Lee et al., 2020). Taken together, these findings indicate the possibility of mitochondrial alterations caused by CN extract on fungi. However, it is completely unknown, to the best of our knowledge, whether a combination of bacterial LPs and CN extract may cause a synergistic antifungal effect against species of the *Fusarium oxysporum* complex, and whether alterations of the bioenergetic response may mediate such effect. Therefore, the aim of this study was to evaluate the effect of a methanolic CN extract and LPs, both as individual agents and as a mixture, against *Foc* strain IB (race1). Furthermore, we investigated the hypothesis that alterations of mitochondrial bioenergetics are part of the antifungal mechanism exhibited by these natural products. To this end, we used high resolution respirometry to determine changes in fungal oxygen consumption rates as a means to perform a bioenergetic discrimination of the effect of bacterial LPs and CN extract on fungal bioenergetics.

Materials and methods

Reagents

Oligomycin A, F_1F_0-ATPase inhibitor; carbonyl cyanide 4- trifluoromethoxy phenylhydrazone (FCCP), mitochondrial uncoupler; and antimycin A from *Streptomyces* sp., complex III inhibitor, were obtained from Sigma-Aldrich. Other reagents such as potassium chloride (KCl), magnesium chloride ($MgCl_2$), and methanol were from Merck. Potassium phosphate dibasic (K_2HPO_4), dextrose, hepes and EGTA were from AMRESCO.

Microorganisms and culture conditions

Bacillus tequilensis EA-CB0015 (NCBI reference sequence NZ_CP048852.1), previously isolated from the phyllosphere of a banana plant in Urabá, Antioquia, Colombia (Ceballos et al., 2012) was activated from frozen cultures on 50% TSA (Tripticase Soy Agar, Merck) for 48 h at 30 °C before any experimental use. For identification of the *Fusarium* isolate, sequence analysis of the RNA polymerase II largest subunit (*rpb1*) and the RNA polymerase II second largest subunit (*rpb2*) were used. Nucleotide sequences were submitted to GenBank and were deposited with accession numbers OR698889 and OR698890, respectively. This isolate was identified as *Foc* IB, a pathogen on Gros Michel (AAA) (Maryani et al., 2019). *Foc* strain IB from Universidad EAFIT was kept at 4 ºC in folded filter paper during storage. Prior to their use for biomass production, the fungal strain was activated by plate culture on Potato Dextrose Agar (PDA, Oxoid) for 7-9 days at 30°C. *Fusarium* spores were resuspended with sterile water and were adjusted to 1.5×10^5 spores·mL^{-1}.

On the other hand, additional experiments were conducted in the Laboratory of Phytopathology at the Wageningen University. To this end, strains of *Foc* IB, *F. phialophorum* CR1.1A (CR1.1A), and *F. odoratissimum* II5 (TR4-II5) were activated on PDA at 25°C for 7 days before growth assays.

Cyclic lipopeptides and cinnamon extract

Production, extraction and purification of cyclic LPs from *B. tequilensis* EA-CB0015 were performed as previously described (Mosquera et al., 2014, Villegas-Escobar et al., 2013). The mixture comprising iturin, fengycin, and surfactin isoforms was kept at 4 ºC until its use.

CN extract was obtained through hydro-distillation, following a previously described methodology (Roohinejad et al., 2017). Briefly, culinary cinnamon sticks (60 g) were immersed in a 350 mL ethanol bath (96%) and heated to boiling temperature. The extract decanted by cooling was evaporated to a solid residue, with a yield of 51 mg cinnamon extract/g cinnamon sticks. When needed, cinnamon extract was diluted in methanol and stored at 4 ºC until any experimental use.

Growth kinetics and culture conditions

Foc IB biomass was produced in liquid culture, using Sabouraud-2% Dextrose Broth (Merck). Briefly, fungal spores were collected from a Petri dish after 7 days of growth and adjusted to 1.5×10^5 spores·mL^{-1} using a Neubauer counting chamber. Erlenmeyer flasks containing 20 mL of liquid media were inoculated with 1 mL of the spore suspension and the corresponding treatment was added (lipopeptides, cinnamon extract or a combination of both). For biomass production assays, the following concentrations were used: 64, 80, 96, 128 and 256 ppm (LPs), and 122 and 152.5 ppm (CN extract). On the other hand, 128 ppm LPs and 152.5 ppm CN extract were the treatments used for respirometry assays. Also, these experiments were done using a mixture comprising 12.8 ppm LPs and 61 ppm CN extract. Treated cultures and the corresponding controls were incubated at 30 ± 0.5 ºC and 120 rpm for 96 h. In all cases, samples were taken every 4 h (for growth kinetics) or 24 h (for oxygen consumption assays). In addition, total biomass within each flask was harvested to determine its dry weight. Growth kinetics parameters were determined by fitting the data to the Gompertz model (Wilkins, 2022). A specific growth rate of 0.4211 h^{-1}, 95% confidence interval [0.2611 h^{-1}, 0.799 h^{-1}], was determined for untreated cultures of *Foc* IB ($R^2 = 0.9639$). All experiments were performed in triplicate.

In vitro inhibitory effect of LPs and CN extract on species of the *Fusarium oxysporum* complex

In order to contrast the inhibitory effect of LPs and CN extract on different species of the *Fusarium oxysporum* complex, which could be expected due to genetic diversity, growth of *F. oxysporum* IB, *F. phialophorum* CR1.1A, and *F. odoratissimum* II5 was evaluated through the dual plate technique, as previously described (Al-Rashdi et al., 2022). It is important to mention that those experiments were done thanks to a collaboration with Dr. Gert Kema, from the Laboratory of Phytopathology at Wageningen University (The Netherlands). This, as our laboratory is not legally allowed to manipulate *Foc*-TR4 due to high risk for banana plantations our Country.

High resolution respirometry assays

Respiratory characteristics of mycelium homogenates were determined by high resolution respirometry using an Oxygraph-2k (Oroboros Instruments) (Gnaiger, 2009; Pesta & Gnaiger, 2012; Porter et al., 2015). Briefly, fungal biomass was filtered using qualitative filter paper (Advantec No.1, diameter 110 mm), recovered in sterile 50 mL tubes, and resuspended in respiration buffer (20 mM Hepes, 5 mM K_2HPO_4, 135 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA and 10 mM dextrose, pH 7.0) (Robles-Martínez et al., 2014). The suspension was gently homogenized using a Dounce tissue grinder (Sigma-Aldrich), and mycelial homogenization was checked under the microscope. A volume of 2 mL of the suspension (40 mg of biomass) was added into each chamber of the Oxygraph-2k. Oxygen consumption rates were obtained using the O2k-software DatLab 7.4 (Oroboros Instruments). ATP-linked respiratory rate, FCCP-stimulated respiratory rate, H^+ leak and non-mitochondrial respiration were determined by successive addition of 60 μM (total) oligomycin A, 6 μM FCCP and 8 μM antimycin A. Those concentrations were previously empirically established to be optimal.

Data analysis

Analysis of variance (ANOVA) was used to analyze each experiment in GraphPad Prism 9.3.0 (GraphPad Software Inc, California, USA). The normality assumption, homoscedasticity, and independence were also determined using the Shapiro-Wilks test, the Levenne's test, and graphic residues vs. run order analysis, respectively. Tukey and Sidak's post hoc tests were applied to assess the significance level for multiple comparisons.

Results

Lipopeptides from *Bacillus tequilensis* EA-CB0015 and cinnamon extract cause growth inhibition of *F. oxysporum* f. sp. *cubense* both as individual agents and in combination.

As shown in Fig. 1A, three growth phases (i.e. lag, exponential and stationary) are evident in liquid cultures of *Foc* IB. As previously stated, a specific growth rate of 0.4211 h^{-1}, 95% confidence interval [0.2611 h^{-1}, 0.7990 h^{-1}], was determined for untreated cultures of *Foc* IB ($R^2 = 0.9639$). In order to determine the effect of different treatments on fungal growth, biomass production was quantified upon 24 h, when cultures were in exponential phase. Analysis of growth kinetics was also done in cultures treated with LPs, CN extract of a mixture of both (data not shown). According to our results, bacterial LPs cause a concentration-dependent decrease in biomass, which was found to be 26% of the corresponding control in cultures treated with 128 ppm LPs (Fig. 1B). Similar results were obtained when fungal cultures were treated with an methanolic CN extract (Fig. 1C). Specifically, biomass production was observed to decline by 56% and 84% in cultures treated with 122 ppm and 152.5 ppm of CN extract, respectively.

On the other hand, when fungal cultures were treated with a mixture composed of 12.8 ppm LPs and 61 ppm CN extract, biomass production was reduced by 80%, relative to control cultures (Fig. 1D). Interestingly, the concentrations of both LPs and CN extract in the mixture were only 10% and 40%, respectively, of the concentrations to which those treatments, individually, caused growth inhibition to a similar extent. Moreover, as seen in the data shown in Table 1, *Foc*-TR4 is slightly less sensitive (44.9% vs

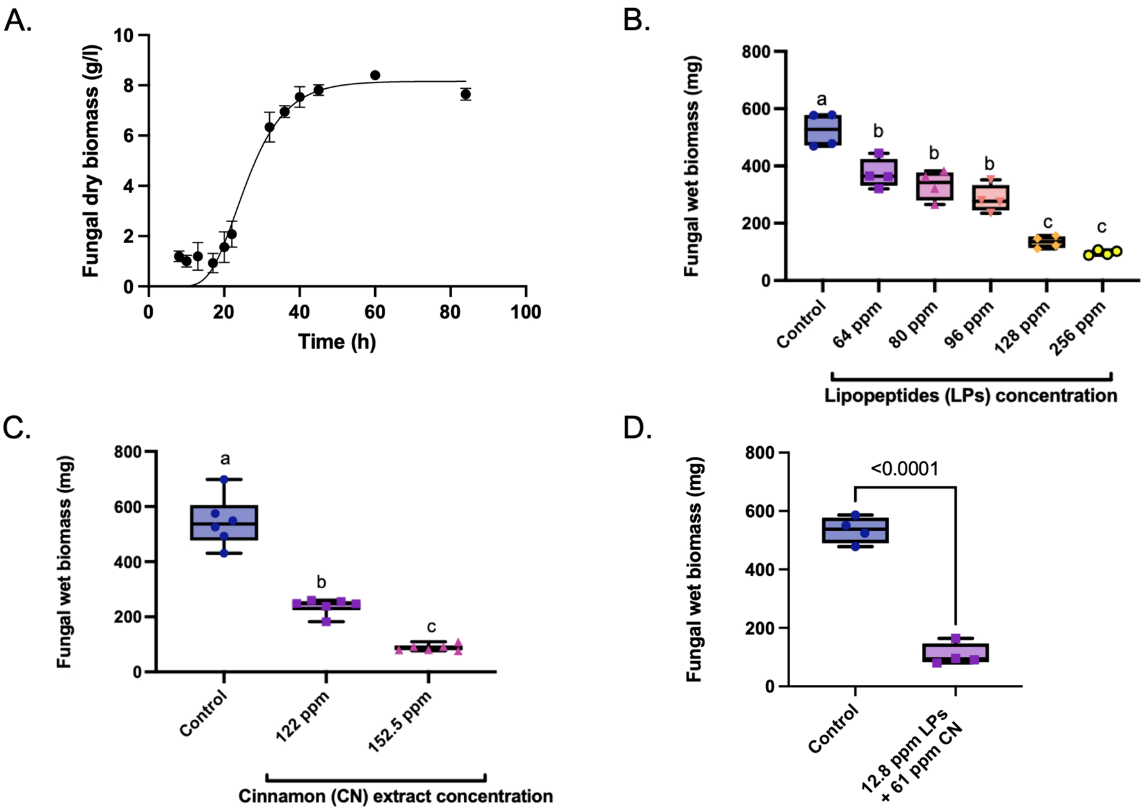

Fig. 1 Fungal biomass production decreases upon treatment of *F. oxysporum* f. sp. *cubense* IB liquid cultures with lipopeptides (LPs) from *B. tequilensis* EA-CB0015 and cinnamon (CN) extract. **(A)** Growth kinetics for control cultures was determined using dry biomass and fitting of the data was done according to the Gompertz model as described in Materials and Methods. Data are shown as the mean ± standard deviation for $n = 3$ independent experiments. **(B-D)** Effect of LPs, CN extract and the mix of both on biomass production after 24 h of growth. Results are shown as box and whisker plots. Different letters above boxes in panels B and C denote a statistically significant decrease ($p < 0.0001$, from a one-way ANOVA) in fungal biomass. The p value shown in panel D was obtained from a two-tailed student's *t*-test. In all cases, symbols within boxes denote the number of independent experiments

55.99%) to growth inhibition caused by LPs, relative to *Foc* IB. On the contrary, there is not significant difference in the inhibitory effect caused by CN extract or the combination of LPs with CN extract.

Treatment of *F. oxysporum* f. sp. *cubense* by lipopeptides from *Bacillus tequilensis* EA-CB0015 and cinnamon extract results in impaired mycelial bioenergetic response.

Our results show that *Foc* IB has a bioenergetic response very similar to that of mammalian cells, which is characterized for an O_2 consumption pattern mostly associated to ATP synthesis (Fig. 2A). As also shown in Fig. 2A, ATP-linked respiratory rate, FCCP-stimulated respiratory rate, H^+ leak and non-mitochondrial respiration can be determined by successive addition of oligomycin A, FCCP and antimycin A. Inhibition by antimycin A was used to determine non-mitochondrial O_2 consumption (Fig. 2A).

Figure 2B shows routine, oligomycin-sensitive, FCCP-stimulated, and antimycin A-sensitive O_2 consumption rates determined in mycelium homogenates from control cultures. According to our data, inhibition of the mitochondrial F_1F_0-ATPase by oligomycin A results, on average, in a 64% decrease of the O_2 consumption rate. On the other hand, the uncoupler FCCP increased the oligomycin A-sensitive O_2 consumption rate by a factor of 3.5, a clear indication of

Table 1 Inhibitory effect of LPs and CN extract on different species of the *Fusarium oxysporum* complex

	F. oxysporum IB	*F. phialophorum* CR1.1A	*F. odoratissimum* TR4-II5
LPs	55.99 ± 2.66 [a]	54.23 ± 3.97 [b]	44.90 ±6.08 [a, b]
CN extract	39.92 ± 0.57	36.13 ± 3.64 [c]	44.72 ± 4.98 [c]
LPs + CN extract	60.14 ± 3.09 [d]	48.35 ± 2.41 [d]	54.45 ± 5.77

Values correspond to the mean percentage of inhibition ± SD for $n = 3$ independent experiments. The percent inhibition for each treatment was calculated considering the fungal growth in the absolute control as 100 %. Treatments correspond to LPs (50 mg/mL, 20 μL), CN extract (50 mg/ml, 20 μL), and a combination of LPs (100 mg/ml, 10 μL) and CN extract (100 mg/ml, 10 μL). Superscripts correspond to p values for comparisons across species within the same treatment

[a] $p = 0.009$

[b] $p = 0.029$

[c] $p = 0.045$

[d] $p = 0.006$

a functional ETC in *Foc* IB. To our surprise, no significant difference was observed between FCCP-stimulated and routine O_2 consumption rates, suggesting the lack of bioenergetic reserve capacity in the exponential phase of fungal growth. In addition, inhibition of mitochondrial complex III by antimycin A was observed to decrease routine and FCCP-stimulated O_2 consumption rates by 81% and 85%, respectively. Therefore, 19% of the routine O_2 consumption rate, on average, appears to be related to non-mitochondrial processes.

With respect to the effect of bacterial LPs or CN extract, our results show a pronounced impairment of the bioenergetic response in all cases (Figs. 3, 4, 5). Based on our data, mycelial routine O_2 consumption rate diminished by 55% (Fig. 3A) and 38% (Fig. 4A) in cultures treated with 128 ppm LPs and 152.5 ppm CN extract, respectively. The same treatments also decrease the FCCP-stimulated O_2 consumption rate by 56% (Fig. 3A) and 40% (Fig. 4A). In order to discern the true extent to which mitochondrial bioenergetics is adversely affected in fungal mycelium treated with LPs or CN extract, changes in ATP-linked respiratory rate, FCCP-stimulated respiratory rate, and H^+ leak were investigated. As shown in Figs. 3B and 4B, 128 ppm LPs and 152.5 ppm CN extract reduced the ATP-linked respiratory rate by 63% and 44%, respectively. Moreover, the same individual treatments of fungal cultures decreased the FCCP-simulated respiratory rate by 63% and 45%, respectively (Figs. 3C and 4C). Therefore, our data suggest that the altered bioenergetic response observed in cultures of *Foc* IB treated with LPs or CN is mainly caused by an impairment of the activity of the ETC components. One experimental result that supports this explanation is the lack of significant differences in the respiration rate corresponding to H^+ leak, when comparing mycelium from treated cultures with their corresponding control (Figs. 3D and 4D). On average, H^+ leak was determined to be 21% of the routine O_2 consumption rate.

Because our analysis of mycelial biomass suggests that there may be a potential synergistic effect of the combination of bacterial LPs and CN extract (Fig. 1), we also investigated the bioenergetic response of mycelium from cultures treated with a mixture composed of 12.8 ppm LPs and 61 ppm. Similar to our results for cultures treated individually with these agents, our data show that, together, they decrease mycelial routine and FCCP-stimulated O_2 consumption rates by 57% and 62%, respectively (Fig. 5A). Furthermore, our results indicate that such a combination diminishes ATP-linked and FCCP-stimulated respiratory rates by 62% and 68%, respectively (Fig. 5B and C). As highlighted in the previous section, interestingly, the concentrations of both LPs and CN extract in the mixture were only 10% and 40%, respectively, of the concentrations to which those treatments, individually, caused growth inhibition and decreased fungal bioenergetic capacity. Finally, although the respiratory rate corresponding to H^+ leak in mycelium from cultures treated with the mixture was found to be significantly different from the corresponding control group (Fig. 5D), those values were so close (29% vs 25% of the routine O_2 consumption rate) that does not seem reasonable to think

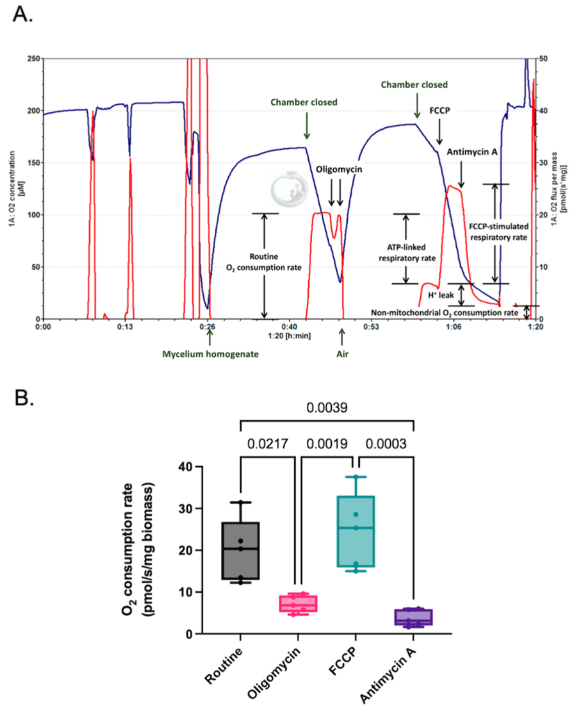

Fig. 2 Oxygen consumption and respiratory characteristics in samples of *F. oxysporum* IB untreated mycelium homogenate. **(A)** Samples were added to each chamber of the Oxygraph-2k system and O_2 concentration (blue trace) and specific O_2 consumption rate (red trace) were determined. As indicated, routine O_2 consumption rate was determined in the closed chamber, following saturation of the respiration buffer. At the time points indicated, oligomycin A (60 µM total) and antimycin A (8 µM) were added as inhibitors of the mitochondrial F_1F_0-ATPase and the complex III, respectively. Also, FCCP (6 µM) was used to uncouple mitochondrial respiration. Traces are representative of at least sixteen independent experiments. All experiments were performed at 25 ºC. **(B)** Routine, oligomycin-sensitive, FCCP-stimulated, and antimycin A-sensitive O_2 consumption rates determined in mycelium homogenate. Results are shown as box and whisker plots for cultures in exponential phase of growth. Numbers above boxes correspond to the p value obtained from a Tukey's post-hoc test together with a one-way ANOVA ($p < 0.0001$). In all cases, symbols within boxes denote the number of independent experiments

on alterations of mitochondrial membrane integrity as the leading cause of the altered bioenergetic response observed.

Discussion

In this study, we shed light on the cellular events underlying the antifungal effect of natural products such as bacterial lipopeptides or the metabolites present in a methanolic cinnamon extract. Specifically, our data support the hypothesis that alterations to mitochondrial bioenergetics play a key role in the effect of these agents against the fungal phytopathogen *F. oxysporum* IB. This hypothesis is also supported by a recent study that demonstrates the key role cell energy status regulation plays in the mycelial development and pathogenicity of *Arthrobotrys oligospora* (Wang et al., 2022). Our detailed analysis of the fungal bioenergetic response reveals a significant decrease in the rate of oxygen consumption, which appears to be strictly related to a deterioration of the ETC components, when mycelium from treated cultures was compared to the corresponding controls. This is because ATP-linked and FCCP-stimulated respiratory rates were affected to a similar extent. In this regard, our results agree well with previous studies where a cinnamon aqueous extract was reported to cause significant inhibition of complex I activity in mitochondria isolated from rat liver (Usta et al., 2003, 2002). Furthermore, a decrease in the mitochondrial membrane potential was also reported by these authors. On the contrary, the activity of the F_1F_0-ATPase was stimulated when isolated mitochondria were incubated with the cinnamon extract in a concentration as high as 37 mM (Usta et al., 2003, 2002). Moreover, cinnamaldehyde, one of the main compounds found in cinnamon (Stevens & Allred, 2022; Tian et al., 2022) was recently reported to significantly inhibit the growth of the mold *Penicillium expansum*, a postharvest pathogen of fruits, at the same time that a diminished mitochondrial membrane potential was observed (Wang et al., 2018). Interestingly, significant downregulation of differentially expressed genes involved in energy-metabolism was also reported in the same study, consistent with an impaired bioenergetic response as on the leading factors mediating the antifungal capacity of cinnamaldehyde.

By determining the specific alterations to fungal bioenergetic response, our results add to previous efforts to discern the cellular events that mediate the antifungal effect of bacterial lipopeptides and cinnamon extract. In this regard, it is worth mentioning one pioneering study in which WH1fungin, a surfactin isolated from *Bacillus amyloliquefaciens* WH1, was found to increase ROS production and activity of caspase-like proteins, a characteristic feature of

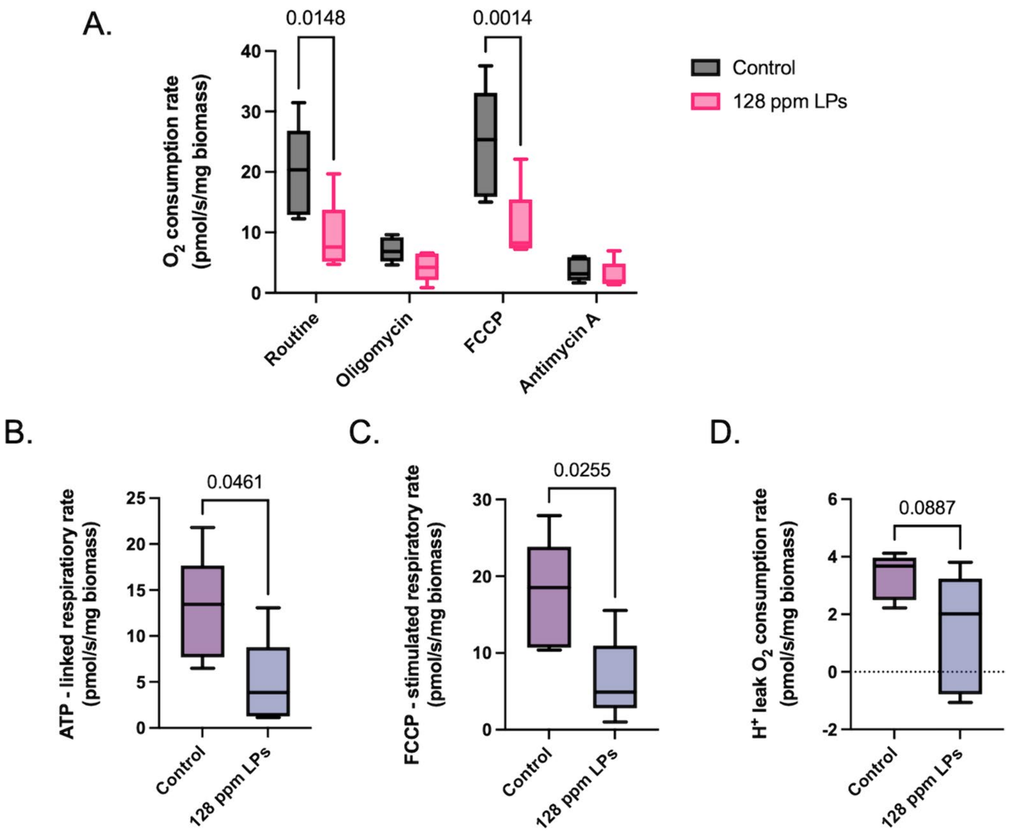

Fig. 3 Bioenergetic response of *F. oxysporum* f. sp. *cubense* strain IB is adversely affected upon treatment of cultures with lipopeptides (LPs) from *B. tequilensis* EA-CB0015. **(A)** Routine, oligomycin-sensitive, FCCP-stimulated, and antimycin A-sensitive O$_2$ consumption rates determined in both control and treated mycelium homogenates (*n* = *5*). Results are shown as box and whisker plots for cultures in exponential phase of growth. Numbers above boxes correspond to the p value obtained from a Sidak's multiple comparison test together with a two-way ANOVA analysis for the effect of lipopeptides ($p = 0.0349$). Analysis of ATP-linked respiratory rate **(B)**, FCCP-stimulated respiratory rate **(C)**, and H$^+$ leak **(D)** (see Materials and methods). The p values shown in panels C-D were obtained from a two-tailed student's *t*-test

programmed cell death which involves mitochondrial dysfunction, as part of its activity against *Rhizoctonia solani* (Qi et al., 2010). In accordance with this, alterations in fungal cell ultrastructure, upon treatment with bacterial lipopeptides, have been extensively reported as well. For instance, hyphal morphology alteration in addition to an increase in the generation of Reactive Oxygen Species (ROS) was identified as the main effect of surfactin species CS30-1 and CS30-2 from *Bacillus* sp. CS30 on *Magnaporthe grisea* (Wu et al., 2019). In a similar study, chromatin condensation, ROS accumulation and lower mitochondrial membrane potential in hyphal cells of *M. grisea* were observed to be induced by fengycins isolated from *Bacillus subtilis* BS155 (Zhang & Sun, 2018).

Also, alteration of conidial and hyphal morphology, together with changes in cell surfaces and cellular contents, plasma membrane integrity and cell wall were reported in *Fusarium graminearum* treated with lipopeptides from *B. amyloliquefaciens* S76-3 (Gong et al., 2015). In addition, alterations of mycelial growth and intracellular ultrastructures were observed in *Botrytis cinerea* treated with lipopeptides from *Bacillus* XT1 CECT 8661 (Toral et al., 2018). On the other hand, similar mycelial alterations have been reported in the filamentous fungus *Podospora anserina* during aging (Osiewacz, 2002; Osiewacz et al., 2010). Therefore, our results could be indicating mitochondrial abnormalities that are reminiscent of fungal aging, as a consequence of bacterial lipopeptides and cinnamon extract. Specifically, alterations in mitochondrial

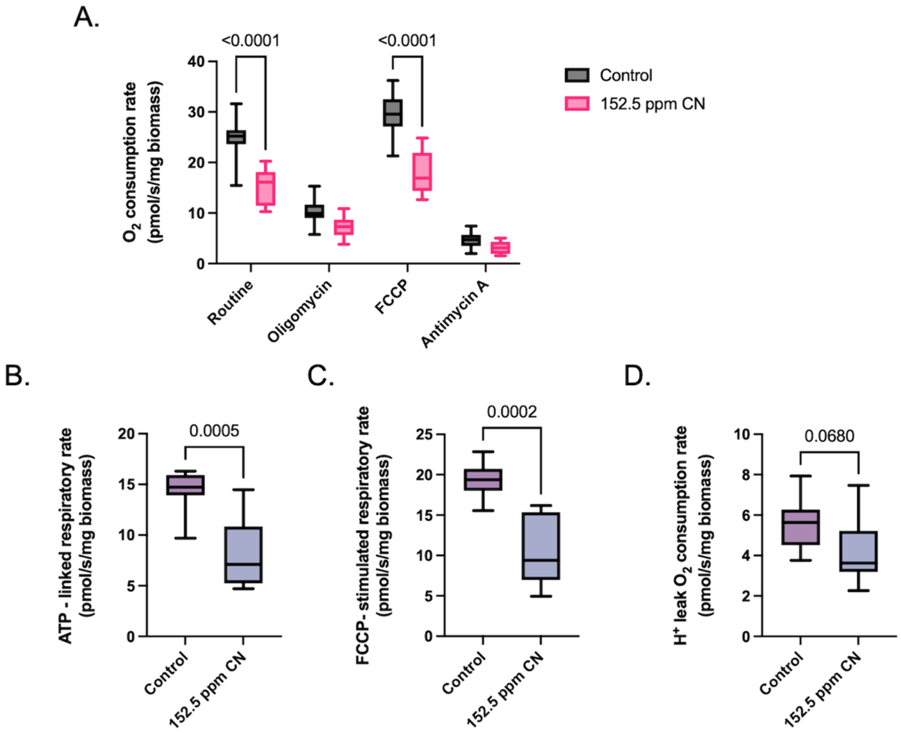

Fig. 4 Bioenergetic response of *F. oxysporum* f. sp. *cubense* strain IB is adversely affected upon treatment of cultures with cinnamon (CN) extract. **(A)** Routine, oligomycin-sensitive, FCCP-stimulated, and antimycin A-sensitive O_2 consumption rates determined in both control and treated mycelium homogenates (*n* = *8*). Results are shown as box and whisker plots for cultures in exponential phase of growth. Numbers above boxes correspond to the p value obtained from a Šidák's multiple comparison test together with a two-way ANOVA analysis for the effect of lipopeptides ($p < 0.0001$). Analysis of ATP-linked respiratory rate **(B)**, FCCP-stimulated respiratory rate **(C)**, and H^+ leak **(D)** (see Materials and methods). The p values shown in panels C-D were obtained from a two-tailed student's *t*-test

dynamics were reported in *P. anserina* mutants lacking the dynamin-related protein 1 (Dnm1p), a protein key to regulate mitochondrial fission (Scheckhuber et al., 2007). Interestingly, those fungal mutants were resistant to induction of apoptosis and increased their life span. Thus, cell death and mitochondrial dynamics are intimately related in filamentous fungi.

Even though alterations of the lipid milieu of fungal biomembranes have been previously reported as the underlying factor of fungal inhibition by bacterial lipopeptides (Fiedler & Heerklotz, 2015; González-Jaramillo et al., 2017; Siahmoshteh et al., 2018), our data does not support that energy uncoupling, mediated by alterations in the inner membrane of mitochondria, plays a significant role in the loss of bioenergetic response. Moreover, our results indicate that lipopeptides from *B. tequilensis* EA-CB0015 and cinnamon extract significantly decrease oxidative capacity because an impairment of the ETC components, which could be hypothesized to be involved in alterations of mitochondrial dynamics.

Conclusion

The role mitochondrial function plays in fungal growth and pathogenicity has been a topic of interest during the last twenty years. While several studies have focused on fungi responsible by infections in humans, attention to mitochondria and energy metabolism in phytopathogens is only being paid more recently. Moreover, specific and detailed studies focused on establishing whether mitochondrial abnormalities are part of the mechanism by which

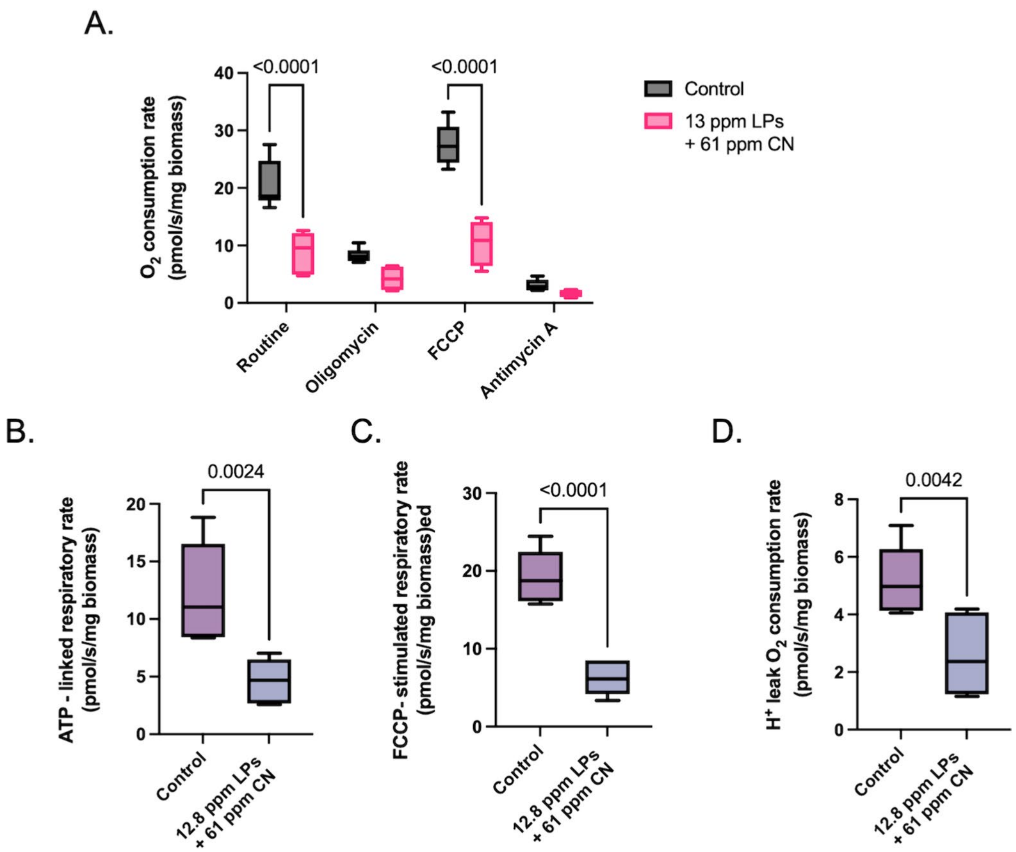

Fig. 5 Bioenergetic response of *F. oxysporum* f. sp. *cubense* strain IB is adversely affected upon treatment of cultures with a mix comprising 12.8 ppm LPs and 61 ppm CN extract. **(A)** Routine, oligomycin-sensitive, FCCP-stimulated, and antimycin A-sensitive O_2 consumption rates determined in both control and treated mycelium homogenates ($n = 6$). Results are shown as box and whisker plots for cultures in exponential phase of growth. Numbers above boxes correspond to the p value obtained from a Šidák's multiple comparison test together with a two-way ANOVA analysis for the effect of lipopeptides ($p < 0.0001$). Analysis of ATP-linked respiratory rate **(B)**, FCCP-stimulated respiratory rate **(C)**, and H^+ leak **(D)** (see Materials and methods). The p values shown in panels C-D were obtained from a two-tailed student's *t*-test

bacterial and plant metabolites affect fungal phytopathogens are barely five years old. In this regard, this work represents, to the best of our knowledge, the first examination of alterations to mitochondrial bioenergetics in *Foc* related to the inhibitory effect of what appears to be a synergistic combination of lipopeptides from *B. tequilensis* EA-CB0015 with a methanolic cinnamon extract. Further work should be addressed in order to gain more insights into the potential applications of this combined treatment.

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Writing – review & editing (LAG, VVE, JMRM). All the authors have read the paper and have agreed to be co-authors.

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Data Availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations 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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