Antifungal activity of lipopeptides isolated from *Bacillus subtilis* EA-CB0015 against *Colletotrichum acutatum* EAHP-008 is not related to an impairment in cellular bioenergetic reserve capacity

Tobón Ospina, Juliana[1,2]; Luis A. Gómez[1]

[1] Departamento de Ciencias Biológicas - Escuela de Ciencias
[2] Programa de Maestría – Escuela de Ingeniería

Abstract

Bacillus subtilis has been instrumental to advance towards novel strategies to control several phytopathogens such as *Colletorichum acutatum.* In this regard, lipopeptides isolated from *B. subtilis* EA-CB0015, a species native from Colombia, were previously reported to have remarkable high antifugal activity. However, the underyling cellular events involved in the mechanism of such antifungal microbial metabolites are not completely understood. Thus, the main goal of this work was to investigate whether the inhibitory effect lipopeptides from *B. subtilis* EA-CB0015 have on *C. acutatum* EAHP-008 is related to an impairment of mitochondrial function. We used high resolution respirometry to determine changes in fungal bioenergetic reserve capacity as an indicator of mitochondrial health and cellular energy metabolism upon treatment of the fungal phytopathogen with lipopeptides in a liquid culture. Our results show that the contribution of the Alternative Oxidase (AOX) pathway to electron transport is minimal during exponential growth, and that the classical electron transport chain (ETC) plays a key role in the generation of proton motive force during this growth phase. Also, we found a shift in the pathway of electron transport from the classical ETC to the AOX pathway, induced by the change in the growth phase of *C. acutatum* EAHP-008. Nevertheless, treatment with lipopeptides of *B. subtilis* EA-CB0015 maintained the predominance of the classical ETC pathway for electron transport in the stationary phase, probably as a result of a bioenergetic compensation. Furthermore, bioenergetic reserve was found to be limited, and treatment of fungal cultures with lipopeptides did not result in a detriment of such spare respiratory capacity, independent of the phase growth.

Key words: *C. acutatum* EAHP-008, *B. subtilis* EA-CB0015, lipopeptides, bioenergetic reserve capacity.

1. Introduction

Colletotrichum species are currently among the fungal phytopathogens of the highest scientific and economical importance (Dean et al. 2012). Specifically, *C. acutatum* is the cause of anthracnose, a disease that adversely affects fruit crops and produces important economical losses as a consequence (Damm et al. 2012, Douanla-Meli and Unger 2017, Gregori et al. 2008). Although agrochemicals have been used as a traditional means to control such fungal pathogen, there is a growing interest in developing new control strategies based on both chemical synthesis (Mondal et al. 2005, Tsikolia et al. 2019) and biological approaches (Arroyave-Toro et al. 2017, Favaretto et al. 2019, Gond et al. 2015). In this regard, lipopeptides isolated from *B. subtilis* EA-CB0015, a species native from Colombia, were found to have remarkable high activity against of *C. acutatum* EAHP-008 (Arroyave-Toro et al. 2017). However, the underyling cellular events involved in the action mechanism of such antifungal microbial metabolites are not yet completely understood.

Evidence from biophysical studies indicates that changes of 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), one of the lipopeptides produced by *B. subtilis* EA-CB0015 (Villegas-Escobar et al. 2013). Also, membrane destabilization and permeabilization was reported to be a direct consequence of the treatment of lipid vesicles with lipopeptides from *B. subtilis* QST713 (Fiedler and Heerklotz 2015). On the other hand, recent studies suggest that the alteration of mitochondrial function may be one underlying factor of the antifungal effect of bacterial lipopeptides (Avenot and Michailides 2010, Cárdenas-Monroy et al. 2017, Nicola et al. 2019, Patkar et al. 2012, Robles-Martínez et al. 2014).

The main goal of the present work was to investigate whether the inhibitory effect lipopetides from *B. subtilis* EA-CB0015 have on growth of *C. acutatum* EAHP-008 is related, at least in part, to an impairment of mitochondrial function. To this end, we used high resolution respirometry to determine changes in fungal bioenergetic reserve capacity as a means to perform a bioenergetic discrimination of the effect of bacterial lipopeptides on fungal bioenergetics. Such analysis of the spare respiratory capacity, determined as the difference between routine respiration and uncoupled (maximal) respiration, has proven to be a very suitable approach to understand mitochondrial health and performance in response to different stimuli that affect the cell (Choi et al. 2009, Dranka et al. 2010, Hill et al. 2009, Nicholls 2009, Sansbury et al. 2011). Furthermore, because fungal mitochondria display two different pathways that mediate electron transport during respiration, namely the 'classical' electron transport chain (ETC), which is also present in plants and animals, and the Alternative Oxidase (AOX) pathway, which is exclusive of plants and fungi (Joseph-Horne et al. 2001, Martins et al. 2011, Vanlerberghe 2013), we also analyzed whether changes in such alternative capacity are part of the fungal response to bacterial lipopeptides.

2. Materials and methods

2.1. Reagents

ATPase inhibitor Oligomycin A, mitochondrial uncoupler carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP), alternative oxidase inhibitor propyl gallate, and Amberlite XAD16® were obtained from Sigma-Aldrich. Other reagents such as potassium chloride (KCl), magnesium chloride ($MgCl_2$), potassium cyanide (KCN), and methanol were from Merck. Potassium phosphate dibasic (K_2HPO_4), dextrose, hepes and EGTA were from AMRESCO.

2.2. Microorganisms and culture conditions

During storage, *Colletotrichum acutatum* EAHP-008 was kept at 4 ºC in folded filter paper. Prior to its use for biomass production, fungal strain was activated by plate culture on Potato Dextrose Agar (PDA by Oxoid) for 7-9 days at 22 °C. On the other hand, *B. subtilis* EA-CB0015 (GenBank accession number KC006063) was stored at -80 °C in Tryptic Soy Broth (TSB by Oxoid) with 20% glycerol added and was activated by plate culture on half strength Tryptic Soy Agar (TSA by Oxoid) for 48 h at 30 °C.

In order to perform high-resolution respirometry assays using intact mycelium, *C. acutatum* EAHP-008 biomass was produced in liquid culture, using Sabouraud-2% Dextrose Broth (Merck). To properly standardize the inoculum, fungal spores were collected from a petri dish after 9-14 days of growth, and the concentration of the suspension was adjusted to $3x10^5$ spores/ml using a Neubauer counting chamber. Flasks containing liquid cultures (20 ml) were kept at 26 +/- 0.5 ºC and 120 rpm using an incubator shaker. Fungal growth kinetics was established by dry weight measurements over time and a specific growth rate of 0.0420 h^{-1}, 95% confidence interval [0.0332 h^{-1}, 0.0507 h^{-1}], was obtained upon fitting the logistic model to the data ($R^2 = 0.9366$).

2.3. Lipopeptides from B. subtilis EA-CB0015

Production, extraction and purification of lipopeptides from B. subtilis EA-CB0015 was 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.

2.4. Treatment of Colletotrichum acutatum EAHP-008 cultures with lipopeptides

Prior to investigate the effect of lipopeptides isolated from *B. subtilis* EA-CB0015 on the bioenergetics of *C. acutatum* EAHP-008, a titration of liquid cultures was performed as previously described (Wiegand, Hilpert, and Hancock 2008). To this end, fungal spores ($3x10^5$ spores/ml) were cultured in microplate wells, each one containing 100 µl of Saboraud broth, and lipopeptides added to a final concentration ranging from 1 ppm up to 1025 ppm. Microplates were incubated for 156 hrs at 26 °C, and optical density at 595 nm was recorded every 12 hrs using an iMark™ Reader (Bio-Rad). Kinetic parameters (i.e.

specific growth rate and biomass production) were obtained upon fitting the logistic model to the data. Three independent experiments were performed, including six replicates for each concentration of lipopeptides used. As negative control, spores were treated with 32 ppm methanol. Based on the growth kinetic results, fungal spores were additionally cultured in flasks as described in section 2.2, and treated with 8, 10, 12, and 14 ppm of lipopeptides. In addition, mycelial harvest times for respirometry assays were also established. In particular, 72 hrs and 192 hrs were used for exponential and stationary phases of growth, respectively.

2.5. High resolution respirometry assays

Respiratory characteristics of intact mycelium were determined by high resolution respirometry using an Oxygraph-2k (Oroboros Instruments) (Gnaiger 2009, Pesta and Gnaiger 2012, Porter et al. 2015). To this end, biomass was filtered using qualitative filter paper (Advantec No.1, diameter 125 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 integrity was checked under the microscope. A volume of 2 ml of the suspension (100 mg of biomass) was added into each of the chambers of the Oxygraph-2k. Oxygen consumption rates were obtained using the O2k-software DatLab (Oroboros Instruments).

2.6. Statistics

All statistical analyses were done using GraphPad Prism 8.1.1. A student's *t*-test was used to evaluate the effect of lipopeptides from *B.subtilis* EA-CB0015 on biomass production by *C. acutatum* EA-PH008 in liquid culture. On the other hand, the interaction between treatment with lipopeptides and respiration status was investigated using a two-way ANOVA. In this case, Tukey and Sidak multiple comparison tests were additionally done, using a 95% confidence level in both cases.

3. Results

3.1. Fungal growth is inhibited by lipopeptides from B. subtilis EA-CB0015

In order to determine to what extent lipopeptides isolated from *B. subtilis* EA-CB0015 affected biomass production by *C. acutatum* EAHP-008, liquid cultures were treated with different concentrations of a mixture comprising Iturin, Fengycin, and Surfactin isoforms previously identified by our group (Villegas-Escobar et al. 2013). As shown in Figure 1, fungal growth was severely inhibited when lipopeptides reached a concentration higher than 10 ppm. Under such conditions, fungal mycelium was hardly seen, even after 72 hrs of culture. Thus, 10 ppm was chosen as the highest concentration of lipopeptides for treating cultures in all further experiments.

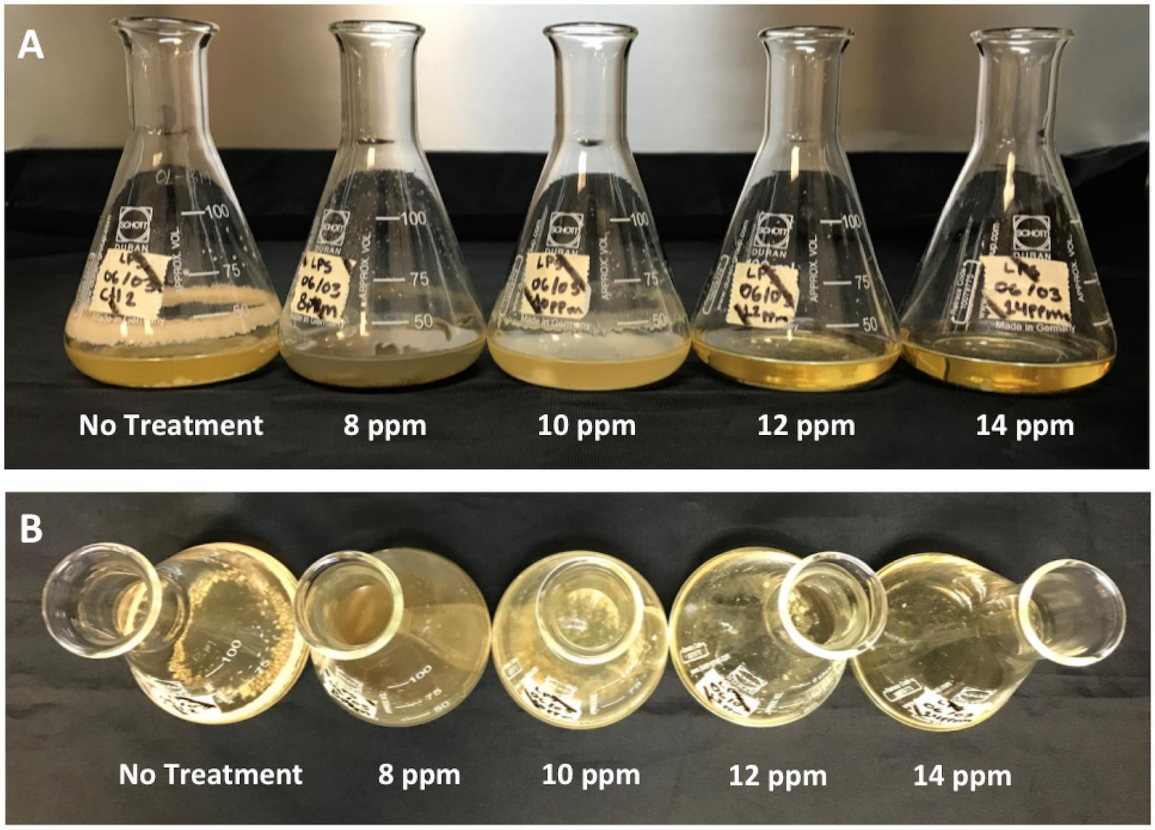

Figure 1. *C. acutatum* EAHP-008 growth upon treatment with lipopetides from *B. subtilis* EA-CB0015. Top (panel A) and side (panel B) views of culture flasks after 72 h of treatment show severe growth inhibition with a lipopeptide concentration greater than 10 parts per million (ppm), relative to the non-treated culture.

With respect to non-treated cultures, we found a significant 44% reduction ($p < 0.0001$) in fungal biomass, from a mean value of 545.8 mg to 303.3 mg, following treatment with 10 ppm of lipopeptides (Fig. 2).

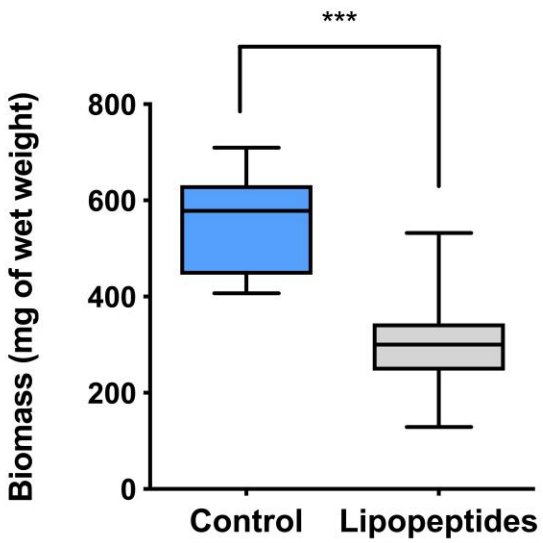

Figure 2. Effect of *B. subtilis* EA-CB0015 lipopeptides on biomass production by *C. acutatum* EAHP-008. Results are shown as box and whisker plots. Asterisks denote a statistically significant decrease ($p < 0.0001$) in fungal biomass after 72 h of growth in the presence of 10 ppm lipopeptides (n = 26), relative to the control group (n = 11). Group comparison was done using a two-tailed student's *t*-test.

3.2. Respiration of C. acutatum EAHP-008 increases upon treatment with lipopeptides from B. subtilis EA-CB0015

To properly establish whether the effect of lipopeptides from *B. subtilis* EA-CB0015 on fungal growth is related, at least in part, to an alteration in mitochondrial bioenergetics, we investigated mycelial respiratory activity. Furthermore, taking into account that, unlike the case in mammalian mitochondria, electron transport in aerobic fungi involves both the cyanide-sensitive ETC, namely the classical ETC, and the AOX pathway, we used inhibitors of such pathways to discern the role they play in respiration of *C. acutatum* EAHP-008. Figure 3 shows typical oxygen consumption traces for both control and mycelium treated with lipopeptides.

In the first place, our results show that for the fungus in both phases of growth there is a statistically significant interaction between the treatment with lipopeptides and the status of mycelial respiration, $F(2,18) = 4.49$ and $p = 0.0261$ in exponential phase, and $F(2,18) = 4.40$ and $p = 0.0278$ in stationary phase, based on a two-way ANOVA (Fig. 4). Very interestingly, our data reveal that treatment of fungal cultures with lipopeptides resulted in a significant (95% confidence level) increase in the mean routine respiration rate of 58% (from 290.5 to 458.4 pmol/s/ml, Fig. 4A) and 89% (from 117.4 to 221.6 pmol/s/ml, Fig. 4B) for the fungus in exponential and stationary growth, respectively.

With respect to the contribution of each electron transport pathway to respiration of *C. acutatum* EAHP-008, our results indicate a surprising difference, depending on whether the fungus is exponentially growing or in the stationary phase. Furthermore, an unexpected

effect on the predominance of a specific electron transport pathway was observed following treatment of the cultures with lipopeptides of *B. subtilis* EA-CB0015.

On the one hand, cyanide caused a significant (95% confidence level) reduction of 64% in the mean routine respiration rate (from 290.5 to 104.2 pmol/s/ml, Fig. 4A) in non-treated cultures in exponential growth. Moreover, subsequent addition of propyl gallate, an AOX inhibitor, to the assay only increased the observed inhibition by 18%. Nevertheless, differences in mean fungal respiration rates in the presence of cyanide or cyanide plus propyl gallate were not found to be statistically significant. Thus, in the presence of both inhibitors, mean routine respiration rate globally decreased by 82% (from 290.5 to 52.3 pmol/s/ml, 95% confidence level), with most of such reduction caused by inhibition of the Complex IV of the classical ETC. On the contrary, when the culture was in the stationary phase, cyanide displayed a very limited capacity to decrease routine respiration and the 37% degree of reduction observed in the mean rate of oxygen consumption (from 117.4 to 73.7 pmol/s/ml) was not statistically significant (Fig. 4B). Furthermore, it was not until both inhibitors; namely, cyanide and propyl gallate, were present that the mean routine respiration rate significantly diminished by 77% (from 117.4 to 26.9 pmol/s/ml, 95% confidence level). Therefore, our results suggest a shift in the pathway of electron transport from the classical ETC to the AOX pathway, induced by the change in the growth phase of *C. acutatum* EAHP-008.

On the other hand, treatment of fungal cultures with lipopeptides resulted in a very different trend with regard to the predominant pathway for electron transport, specifically in relation to the shift observed with the growth phase. Similar to the results obtained for cultures under no treatment, cyanide significantly inhibited mean routine respiration by 73% (from 458.4 to 124.3 pmol/s/ml, 95% confidence level) in cultures exponentially growing (Fig. 4A). In addition, such inhibition was only increased up to 87% (from 458.4 to 61.0 pmol/s/ml, 95% confidence level) in the presence of both cyanide and propyl gallate. However, we found that cultures in the stationary phase also displayed a tendency to use the classical ETC as the main electron transport pathway, based on the 62% decrease in mean routine respiration induced by cyanide (from 221.6 to 85.0 pmol/s/ml, 95% confidence level, Fig. 4B). Furthermore, in the presence of both cyanide and propyl gallate we observed a total inhibition of 84% (from 221.6 to 38.9 pmol/s/ml, 95% confidence level). Thus, lipopeptides of *B. subtilis* EA-CB0015 resulted in a predominance of the classical ETC pathway for electron transport in the stationary phase of *C. acutatum* EAHP-008, which is reminiscent of the bioenergetic pattern seen in exponential growth.

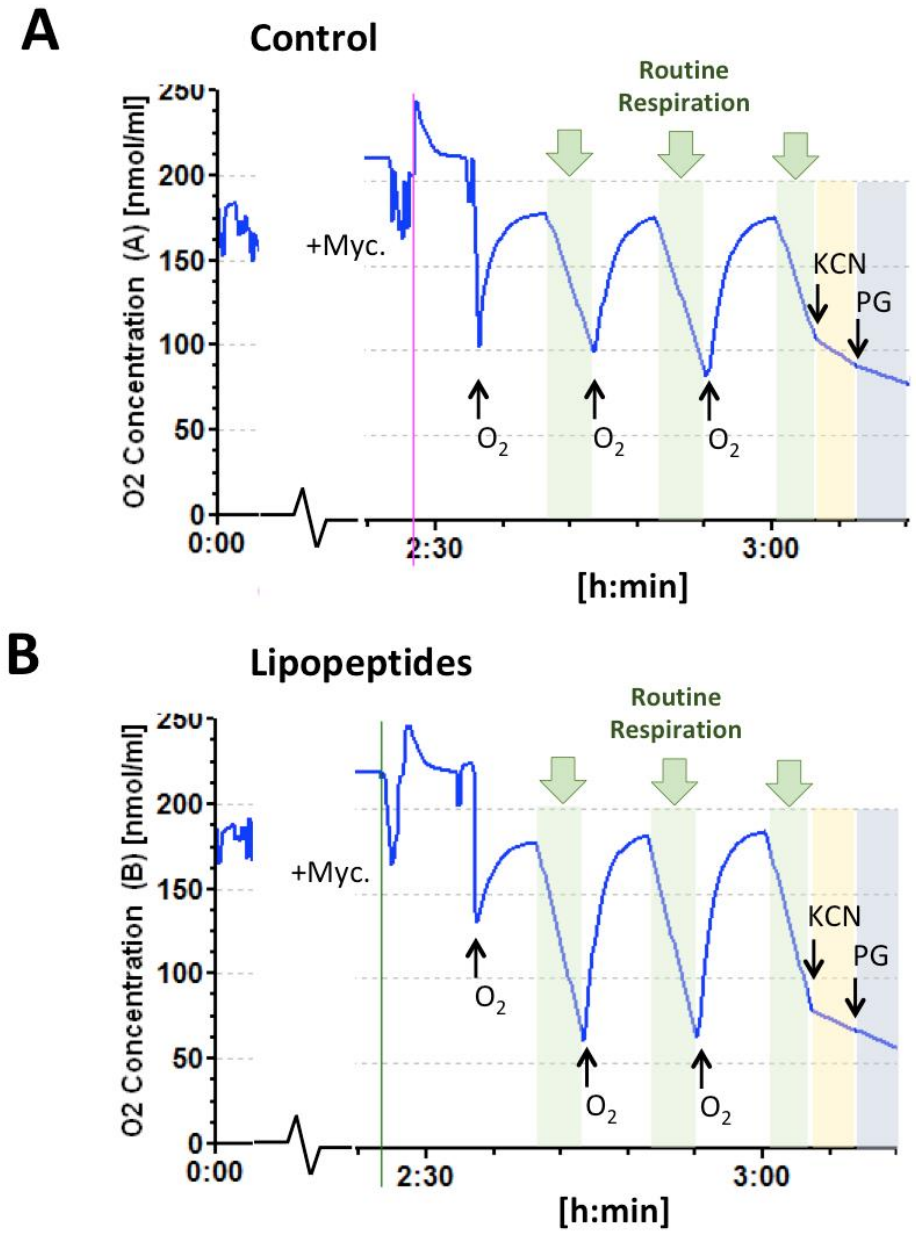

Figure 3. Oxygen consumption traces for intact mycelium of *C. acutatum* EAHP-008 in the exponential phase of growth (panel A; control culture, and panel B; lipopeptide-treated culture). 100 mg of fungal mycelium (Myc.) was added to each chamber of the Oxygraph-2k system and routine respiration was determined as the oxygen consumption rate in the closed chamber, following saturation of the respiration buffer as indicated. Also, at the time points indicated, 250 µM potassium cyanide (KCN) and 250 µM propyl gallate (PG) were added as inhibitors of the mitochondrial Complex IV and the alternative oxidase (AOX), respectively. Oxygen consumption traces are representative of four independent experiments. All experiments were performed at 25 ºC.

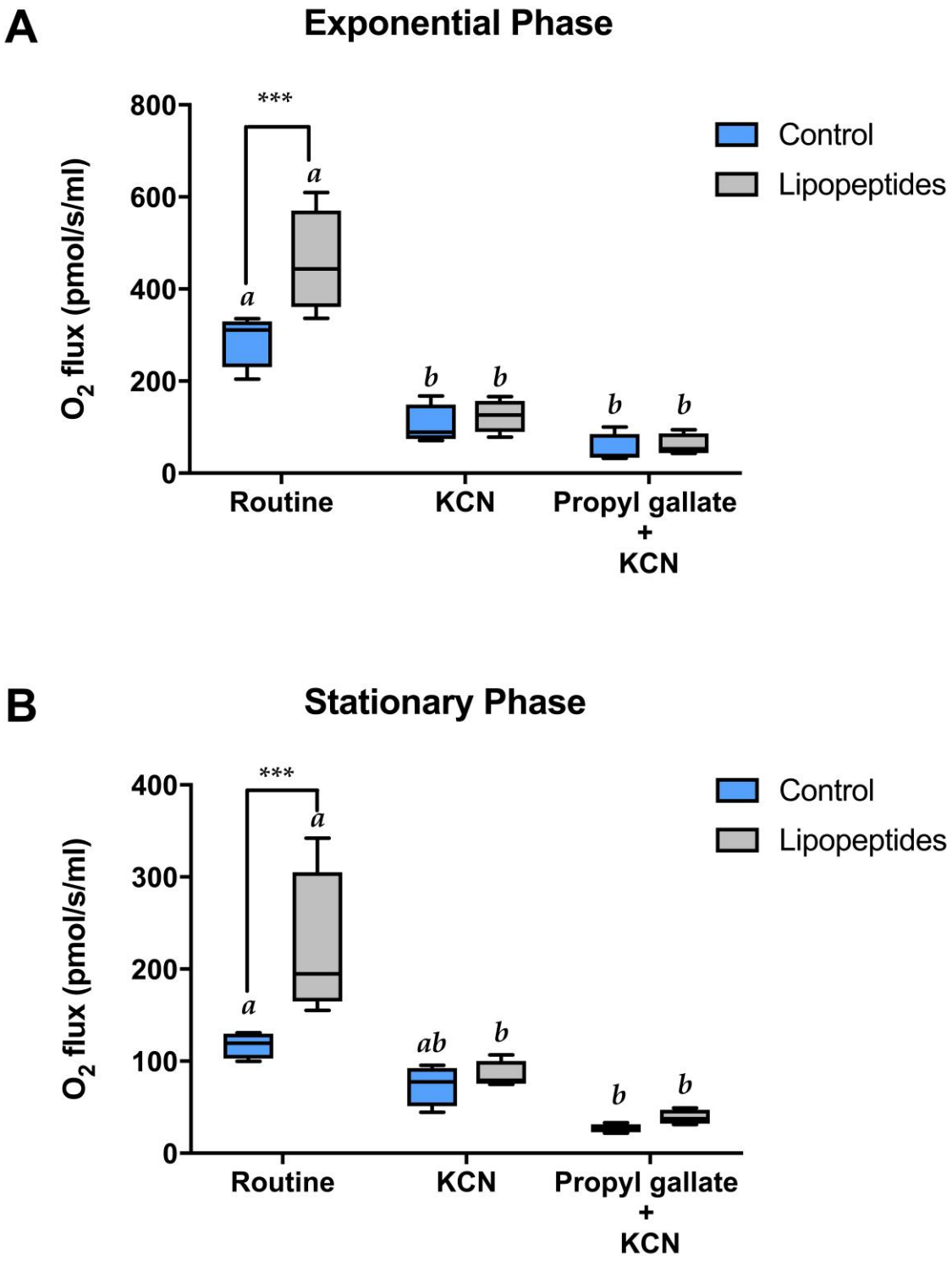

Figure 4. Differential contribution of the classical electron transport chain (ETC) and the cyanide-resistant AOX pathway to cellular respiration of *C. acutatum* EAHP-008, and its

modulation by lipopeptides from *B. subtilis* EA-CB0015. Oxygen consumption rates by intact mycelium (100 mg) were determined in both control cultures and lipopeptide-treated cultures (n = 4). Results are shown as box and whisker plots for cultures in exponential phase of growth (panel A) and stationary phase of growth (panel B). Statistical significance of the interaction between treatment with lipopeptides and respiration status was determined using a two-way ANOVA [$F(2,18) = 4.49$ and $p = 0.0261$ for results in panel A, and $F(2,18) = 4.40$ and $p = 0.0278$ for results in panel B]. A Sidak's test was used for assessing the effect of lipopeptides on cellular respiration, and a Tukey's test for comparison of different respiration rates within the same treatment group (i.e. control or lipopeptides). Within the same treatment group, different letters denote significant differences ($\alpha = 0,05$). Asterisks denote a significant difference ($\alpha = 0,05$) between lipopeptide-treated cultures and control cultures. Potassium cyanide (KCN) and propyl gallate were used as inhibitors of the mitochondrial Complex IV and the AOX pathway, respectively.

3.3. Growth inhibition of C. acutatum EAHP-008 by lipopeptides is not related to an impairment in bioenergetic reserve capacity

To further investigate the effect of lipopeptides on bioenergetics of *C. acutatum* EAHP-008, we performed an assessment of cellular respiration under the concept of bioenergetic reserve capacity. That is, the difference between routine and uncoupled (maximal) respiratory activity. Such experimental approach is instrumental to examine the oxidative phosphorylation process discriminating all the key aspects of energy coupling (Fig. 5), with the further advantage of using intact cells.

As shown in Figure 6, there is a statistically significant interaction between the treatment with lipopeptides and the status of mycelial respiration, $F(3,32) = 3.42$ and $p = 0.0290$ in exponential phase, and $F(3,32) = 3.32$ and $p = 0.0320$ in stationary phase, based on a two-way ANOVA. Specifically, such interaction is mainly related to the significant (95% confidence level) increase in the mean routine respiration rate observed in exponential growth (61.5%, from 323.2 to 521.9 pmol/s/ml) as well as in stationary phase (81%, from 132.1 to 239.7 pmol/s/ml). Although a significant increase of 71% was also found in the mean rate of FCCP-stimulated respiration in stationary phase (from 172.0 to 294.4 pmol/s/ml).

In addition, there are three important findings to highlight from our results, with respect to the effect of lipopeptides on the bioenergetics of *C. acutatum* EAHP-008. First, inhibition of ATP synthesis by oligomycin significantly (95% confidence level) decreased the mean routine respiration rate by 45% (from 323.3 to 177.6 pmol/s/ml) and 67% (from 521.9 to 170.5 pmol/s/ml) in control and treated cultures exponentially growing, respectively (Fig. 6A). Thus, lipopeptides from *B. subtilis* EA-CB0015 do not uncouple respiration from oxidative phosphorylation. A similar result was obtained for cultures under treatment upon reaching the stationary phase of growth. That is, the mean routine respiration rate diminished by 53% (from 239.7 to 112.9 pmol/s/ml, 95% confidence level) upon inhibition of ATP synthesis by oligomycin. On the contrary, non-treated cultures in stationary phase showed a very modest inhibition of the mean routine respiration upon addition of

oligomycin, which most likely reflects a quiescent metabolic status (Fig. 6B). Second, addition of FCCP, which acts as a direct uncoupler, resulted in a significant increase (up to maximal) in the mean respiration rate in all cases, indicating preservation of the functional character of the ETC. On the one hand, our data show a 2.4-fold (from 177.6 to 432.8 pmol/s/ml, 95% confidence level), and a 3.1-fold (from 170.5 to 528.7 pmol/s/ml, 95% confidence level) increase in the mean respiration rate of exponentially growing control and treated cultures, respectively. On the other hand, a 1.9-fold (from 91.2 to 172.0 pmol/s/ml, 95% confidence level), and a 2.6-fold (from 112.9 to 294.4 pmol/s/ml, 95% confidence level) increase in the mean respiration rate was observed for control and treated cultures in the stationary phase. Third, of special interest is the fact that neither cultures exponentially growing nor cultures in stationary phase showed a statistically significant difference between the mean rates of routine and uncoupled (i.e. maximal) respiration. Therefore, our data show that in *C. acutatum* EAHP-008 bioenergetic reserve is limited, and that treatment of fungal cultures with lipopeptides does not result in a detriment of such spare respiratory capacity, independent of the phase growth. In all cases, uncoupled respiration was significantly inhibited by cyanide. That is, 80% inhibition (from 432.8 to 86.6 pmol/s/ml), and 73% inhibition (from 528.7 to 142.5 pmol/s/ml) for control and treated cultures exponentially growing, respectively. In addition, 66% inhibition (from 172.0 to 59.3 pmol/s/ml), and 72% inhibition (from 294.4 to 83.5 pmol/s/ml) for control and treated cultures in stationary phase, respectively

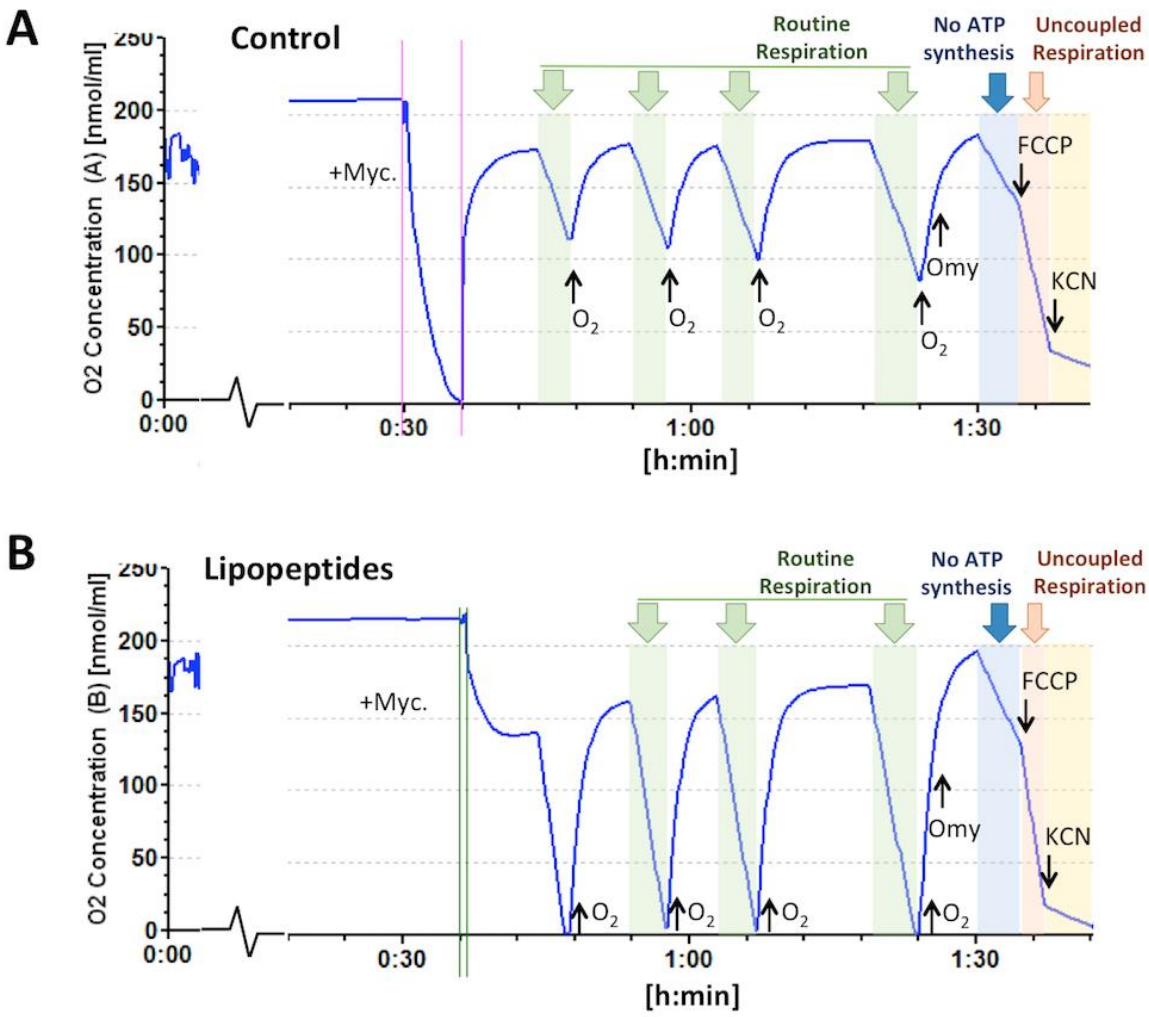

Figure 5. Oxygen consumption traces for intact mycelium of *C. acutatum* EAHP-008 in the exponential phase of growth (panel A; control culture, and panel B; lipopeptide-treated culture). 100 mg of fungal mycelium (Myc.) was added to each chamber of the Oxygraph-2k system and routine respiration was determined as the oxygen consumption rate in the closed chamber, following saturation of the respiration buffer as indicated. To determine oxygen consumption rates not related to ATP synthesis (i.e. proton leak), 8 µM Oligomycin A (Omy) was added to the assay. Uncoupled respiration was obtained by adding 4 µM FCCP. Electron transport through the ETC was inhibited by adding 250 µM KCN. Oxygen consumption traces are representative of five independent experiments. All experiments were performed at 25 ºC.

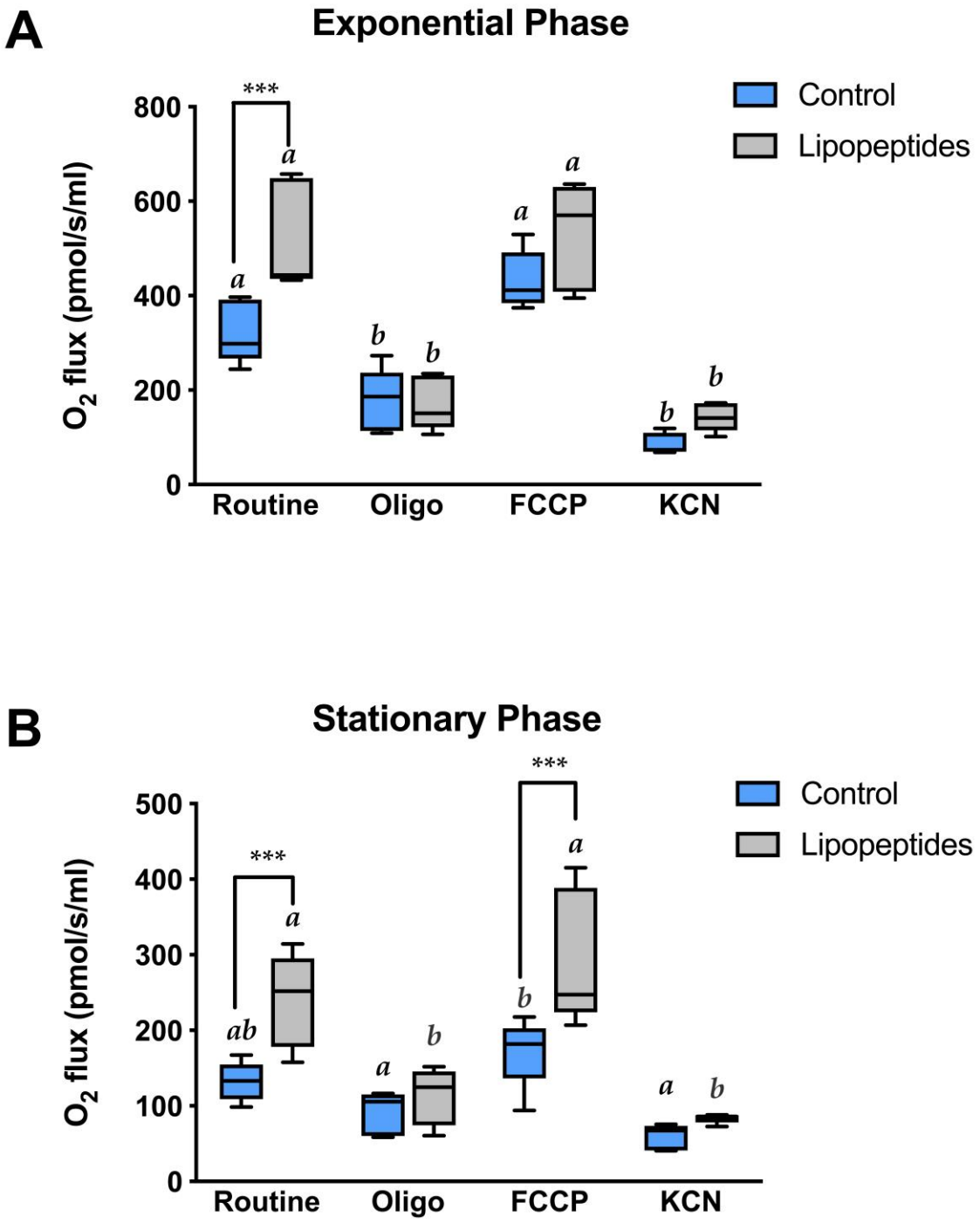

Figure 6. Cellular respiration and bioenergetic reserve capacity of *C. acutatum* EAHP-008 upon treatment of fungal cultures with lipopeptides from *B. subtilis* EA-CB0015. Oxygen consumption rates by intact mycelium (100 mg) were determined in both control cultures and lipopeptide-treated cultures (n = 5). Results are shown as box and whisker plots for cultures in exponential phase of growth (panel A) and stationary phase of growth (panel B). Statistical significance of the interaction between treatment with lipopeptides and

respiration status was determined using a two-way ANOVA [$F(3,32) = 3.42$ and $p = 0.0290$ for results in panel A, and $F(3,32) = 3.32$ and $p = 0.0320$ for results in panel B]. A Sidak's test was used for assessing the effect of lipopeptides on cellular respiration, and a Tukey's test for comparison of different respiration rates within the same treatment group (i.e. control or lipopeptides). Within the same treatment group, different letters denote significant differences ($\alpha = 0,05$). Asterisks denote a significant difference ($\alpha = 0,05$) between lipopeptide-treated cultures and control cultures.

4. Discussion

Bacterial lipopeptides have proven to be instrumental in advancing towards an effective control of fungal phytopathogens (Gong et al. 2015, Rajaofera et al. 2018, Toral et al. 2018, Wu et al. 2019). Nevertheless, the underlying cellular events that are fundamental to understand their antifungal potency are not yet fully understood.

The main goal of the work presented here was to discern whether alterations in mitochondrial bioenergetics explain, at least partially, the inhibitory effect of lipopeptides isolated from *B. subtilis* EA-CB0015 on growth of *C. acutatum* EAHP-008. Very interestingly, we found that treatment with lipopeptides does indeed alters fungal respiration. Nevertheless and contrary to our initial hypothesis, our results show that mitochondrial respiratory capacity is no impaired as a consequence of the treatment of fungal cultures with bacterial lipopeptides. Moreover, a remarkable finding of this study is that, unlike to what has been observed in mammlian cells (Dranka et al. 2010, Hill et al. 2009, Sansbury et al. 2011), fungal growth demands maximal respiratory activity. In addition, we also found that such status of maximal respiratory activity is even preserved in cells treated with lipopeptides. Based on our data it is also clear that such highly-demanding energy status is mainly supported by the classical ETC pathway for fungal cells exponentialy growing, and a shift to the AOX pathway is induced by the change to stationary growth. Consistent with our results, specific induction of the AOX pathway in stationary phase was recently reported in *Ustilago maydis* (Cárdenas-Monroy et al. 2017). Thus, there appears to be a fine regulation of fungal AOX capacity depending on the energy needs of the cell. Very surprisingly, we found that although lipopeptides from *B. subtilis* EA-CB0015 did not alter bioenergetic reserve, neither for cells exponentially growing nor for cells in stationary phase, there was an effect on the AOX capacity. That is, our data show that under treatment with lipopeptides such shift in the pathway for electron transport is prevented, which could be interpreted as a bioenergetic compensatory response.

Because our results negate a detrimental effect of lipopeptides on fungal mitochondrial function, it is important to go deeper into the potential implications of our findings. In this respect, it is worth noting that fungi seem to have the ability to undergo adaptations in mitochondrial function, suggesting a very interesting case of what could be named "mitochondrial plasticity". For instance, mutant strains of *Podospora anserina* lacking mitochondrial Complex IV were reported to use the AOX pathway for electron transport as a compensation and concomitantly increased their respiration rate as a result from a higher mitochondrial content (Scheckhuber et al. 2011). In the mentioned study, a decrease in the ATP content was also reported although was not attributed to apparent changes in

energy coupling. On the contrary, the increase in respiratory activity was explained as a result of changes in respiration rate and mitochondrial content, namely, mass-specific mitochondrial respiration. Another example is the overexpression of Complex IV and AOX observed in mutants of the pathogen yeast *Candida albicans*, as an adaptation to circumvent the lack of Complex I and mantain survival (She et al. 2018). In line with such findings, it is reasonable to hypothesized that *C. acutatum* EAHP-008 maintains a high respiratory status in response to lipopeptides from *B. subtilis* EA-CB0015, at least with concentrations up to10 ppm as used in the present study. Whether such increase in respiration is also concomitant with a change in ATP levels is beyond of the scope of this work. However, it is very tempting to make that hypothesis that lipopeptides may affect mitochondrial dynamics and that is the reason why global respiration and growth are affected when fungal survival is still observed. That is, similar to what has been observed in *P. anserina,* respiratory activity could be increasing as a result of higher mitochondrial content. Such parameter was not specifically examined in our study but alterations in fungal cell ultrastructure upon treatment with bacterial lipopeptides have been extensively reported. 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). Also, alteration of conidial and hyphal morphology, toghether 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. Specifically, alterations in mitochondrial 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 and Heerklotz 2015, González-Jaramillo et al. 2017, Siahmoshteh et al. 2018), our data show that coupled respiration is preserved in treated cultures, which would exclude the possibility of disruption in the lipid organization of mitochondrial membranes. However, our findgins indicate that lipopeptides from *B. subtilis* EA-CB0015, depending on their concentration, could be altering mitochondrial dynamics, specifically favoring fission over fusion events, which could be an attempt to prevent cell death by apoptosis (Fig. 7). To the best of our knowledge, this work represents the first examination of mitochondrial alterations of fungal cultures in relation to treatment with lipopeptides, using a bioenergetic discrimination. Nevertheless, further work should be addressed to gain more insights into the mechanism by which bacterial lipopeptides inhibit fungal growth.

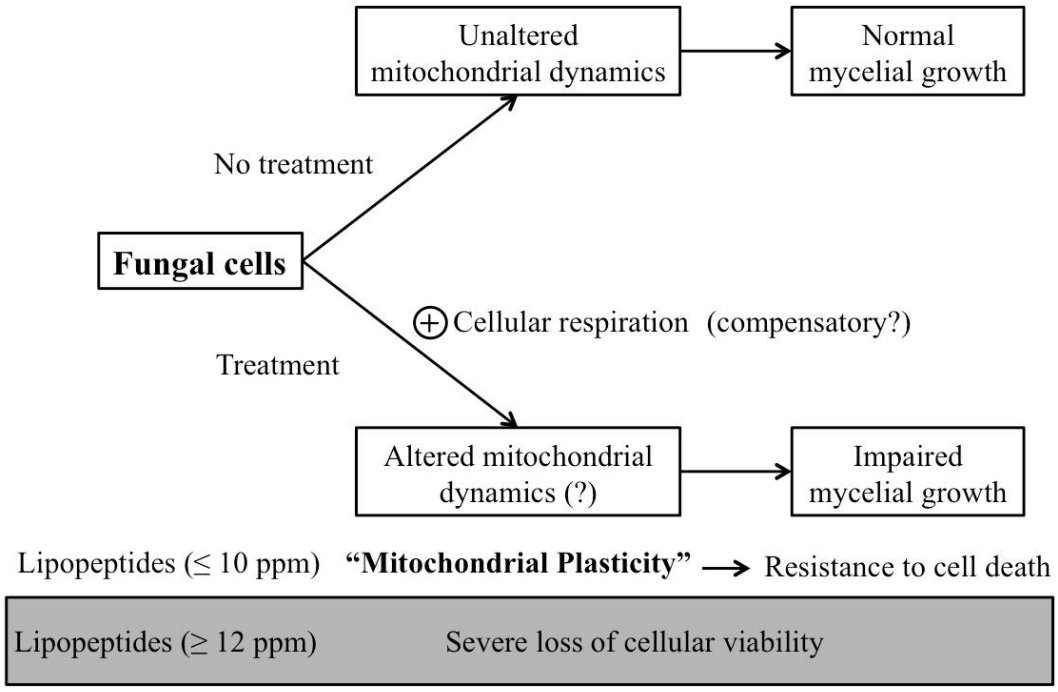

Figure 7. Proposed scheme explaining the role mitochondrial function plays in fungal response to treatment with bacterial lipopeptides.

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