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Ethylene stimulates growth and affects fatty acid content of *Synechocystis* sp. PCC 6803

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A B S T R A C T

This set of results shows that the growth of wild type *Synechocystis* sp. PCC 6803 was enhanced by exogenous ethylene and inhibited by 1-methylcyclopropene (1-MCP), a specific inhibitor of ethylene receptors. The fact that the growth of a *Synechocystis* sp. PCC 6803 strain with the ethylene receptor deleted was unaffected by exogenous ethylene, brings additional proof that this is a specific effect of ethylene. The results also confirm previous observations regarding the positive impact of ethylene on the photochemical efficiency of PSII. Additionally, it was observed that exogenous ethylene enhanced accumulation of C16:0 and C18:0 and C18:1 in the wild type strain. Finally, observations were performed regarding the capacity of the wild type strain to biosynthesize ethylene in the culture medium in the presence of methionine. These results and the recent description of an ethylene receptor in *Synechocystis* should lead to new areas of research in the field of microalgae.

1. Introduction

Recently, Lacey and Binder [1] have shown that *Synechocystis* sp. PCC 6803 harbours a functional ethylene receptor, SynEtr1. These authors showed that ethylene affects phototaxis and PSII levels in *Synechocystis*, which matches previous research describing SynEtr1 as a photoreceptor involved in phototaxis [2], thus Lacey and Binder suggests that SynEtr1 may be a dual input receptor for both light and ethylene.

Lacey and Binder [1] used small amounts of exogenous ethylene, from 0.3 to 1 ppm, to generate these responses. Such ethylene concentrations in water are physiological; indeed, ethylene concentrations of > 20 ppm were measured in flood water of rice ponds [3]. This ethylene may be produced by degradation of organic matters [4] or plant roots [5]. For the moment, no study reports the effect of the 1-methylcyclopropene (1-MCP), a specific inhibitor of ethylene receptors [6], in *Synechocystis*.

Most prokaryotes which are producing ethylene use a pathway that is different from plants. For example, *Pseudomonas syringae* produces

ethylene using 2-oxoglutarate as a substrate ([8] and refs herein). Some other organisms, like *Botrytis* produce ethylene via the oxidation of α -keto γ -methylthiobutyric acid, abbreviated as KMBA, with light or oxygen radicals [9]. For all systems, a common precursor is L-methionine. In plants, methionine is metabolized into S-adenosyl-L-methionine, then 1-aminocyclopropane-1-carboxylic acid (ACC) as intermediates before ethylene [10].

The roles of ethylene in growth of bacteria and fungi have been rarely studied. One interesting article showed that ethylene has a positive impact on growth of the mycorrhizal fungi, *Gigaspora ramisporophora* and *Glomus mosseae*, at very low doses, around 0.01 to 0.1 ppm [11]. In this article, the authors did not report any ethylene impact on growth at higher doses, so this impact might have been missed by other researchers, particularly as ethylene doses are more commonly tested above 1 ppm. A recent study [12] showed that the growth of *Chlorella vulgaris* was not affected by concentrations of 50 and 200 μ M of ethephon, an ethylene precursor. In their conditions (500 ml Erlenmeyer flasks with 200 ml culture medium), if ethephon was completely converted to ethylene, this should represent concentration of 750 to

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3000 ppm of ethylene in the Erlenmeyer flask headspace, but ethylene was not measured in their experiments. However, these researchers found that ethephon affected the content of some fatty acids, and because this is of importance for several industrial applications [13], we decided to follow this effect in our model system.

Regarding wild type *Synechocystis*, Lacey and Binder failed to detect ethylene production in these cyanobacteria. Guerrero et al. [7] also observed that wild type *Synechocystis* failed to produce measurable levels of ethylene, but were able to boost ethylene production by genetically engineering *Synechocystis* with an ethylene-forming enzyme (EFE) from *Pseudomonas syringae*; this excess ethylene did not impact growth. Lacey and Binder [1] suggest that *Synechocystis* may use ethylene as a signal to move towards light as the degradation of organic compounds by light produces ethylene.

Here we report the first evidence that ethylene can mediate growth of *Synechocystis* PCC 6803, and that fatty acid accumulation is affected by various doses of exogenous ethylene and 1-MCP. Additionally, we observed that the *Synechocystis* PCC 6803 may be involved in the production of ethylene depending on the medium and culture environment.

2. Materials and methods

2.1. Cell culture and ethylene or 1-MCP treatment

Synechocystis sp. PCC 6803 strain was obtained from Dr. Martin Hagemann's lab and the Δ SynEtr1 strain, knock-out for the ethylene receptor, has been described in a recent paper [1]. The growth conditions were adapted from Klähn et al. [14] with the following modifications. Cells were grown in 100 ml BG-11 liquid culture containing 5 mM NaHCO_3 in 250 ml Erlenmeyer flasks placed in a rotary shaker at 120 rpm, equipped with a rubber stopper. Conditions of growth were a light intensity of $80 \mu\text{E}/\text{m}^2/\text{s}$ on a 16 h: 8 h light/dark cycle at $27^\circ\text{C} \pm 2^\circ\text{C}$. The initial 730 nm OD value was established around 0.1 by dilution in fresh BG11, and the cells were exposed to 0, 0.01, 0.1, 1, 10 or 100 ppm of ethylene or 0, 0.01, 0.1, 1, 10 ppm of 1-MCP. For each condition, cells were sampled at 0, 24, 48, 72 and 96 h, then the headspace treatment with ethylene and 1-MCP was re-established after 5 min aeration. For the subsequent analyses, the remaining algal cells were harvested by centrifugation at the end of the fifth day of the growth period, and stored at -20°C .

2.2. Ethylene measurements

To check the concentrations used in exogenous ethylene treatments, headspace gas samples were analyzed daily for ethylene by FID GC, as previously described [15]. For measuring the endogenous ethylene production 3 ml of *Synechocystis* cultures were incubated the fourth day of growth period in 9 ml vials plugged with a silicone stopper, in similar growing conditions as described above. Methionine was used at a 10 mM concentration, as described in Chague et al. [9]; for darkness experiments, the vials were wrapped in aluminum foil; for experiment without cells, the cells were harvested by centrifugation at 13,000 g for 15 min, before incubation in the same conditions as described above.

2.3. Fluorescence measurements

Chlorophyll fluorescence was performed with a pulse-amplitude modulation fluorimeter (PAM-2000, Walz, Effeltrich, Germany) on cells (1 ml cell suspensions placed in a stirred cuvette at $\sim 2 \times 10^4$ cells/ μl concentration) adapted to darkness for 20-min. The minimum fluorescence (F_0) and the maximum fluorescence (F_m) following a saturating light pulse were measured as for Touloupakis et al. [16]. The ratio between variable and maximum fluorescence (F_v/F_m where $F_v = F_m - F_0$) was calculated which can be used to estimate the potential photochemical efficiency of photosystem II [17].

2.4. Lipid extraction and analysis

Cell lipids were extracted according to the chloroform + methanol-based method by Folch et al. [18], as it showed the highest lipid recoveries [19], with the following modifications. Forty milligrams of fresh algal biomass and the heptadecanoic acid, used as internal standard, were homogenized with 1 ml of a mixture of chloroform/methanol (2:1) and placed in a rotary shaker at 130 rpm, for 24 h, at room temperature. The mixture was then centrifuged at $5000 \times g$ for 10 min and the supernatant recovered. Solvents were evaporated under nitrogen flow and then placed in a ventilated oven to a constant mass. The lipid extract was finally filtered through a $0.45\text{-}\mu\text{m}$ PTFE membrane. Algal free fatty acids (FAs) and triacylglycerols were transformed into their corresponding methyl esters (FAMES) according to the NFT60-233 standard method: 20 mg of lipid extract were solubilized in 1 ml TBME, and then 100 μl of this solution was mixed with 50 μl of TMSH solution ($0.5 \text{ mol} \cdot \text{l}^{-1}$ in methanol). FA methyl esters from each sample were analyzed by GC according to Valentin et al. [20].

2.5. Statistical data analysis

All data were analyzed with ANOVAs to allow multiple comparison with Tukey's tests. All growth kinetics with different ethylene and MCP doses were performed in four biological replicates.

3. Results and discussion

3.1. Ethylene perception mediates *Synechocystis* growth

As ethylene perception was recently demonstrated to affect *Synechocystis* physiology [1], we tested whether or not the perception of ethylene was affecting *Synechocystis* growth (Fig. 1). We applied five different ethylene headspace concentrations, and also four different headspace concentrations of an inhibitor of the ethylene receptor, 1-methylcyclopropene (1-MCP) [6]. Ethylene significantly stimulated the growth of *Synechocystis* sp. PCC6803 at all ethylene doses. After five days, the best enhancing dose was 0.01 ppm, but there was no

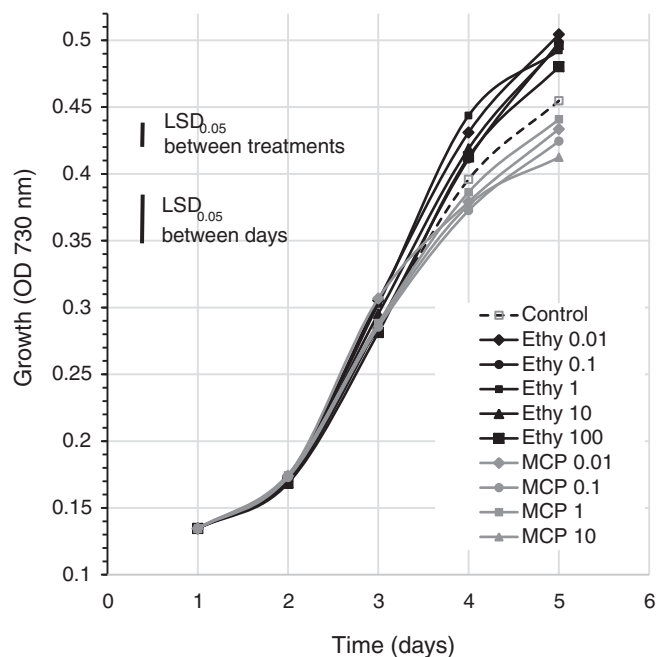


Fig. 1. Effects of various ethylene ("Ethy") or 1-methylcyclopropene ("MCP") treatments on the *Synechocystis* growth measured by absorbance at 730 nm. The various doses ("0.01, 0.1, 1, 10 and 100") are indicated in ppm. The points are means of 4 biological replicates. The LSDs (Least Significant Differences) were calculated at the 0.05 confidence level.

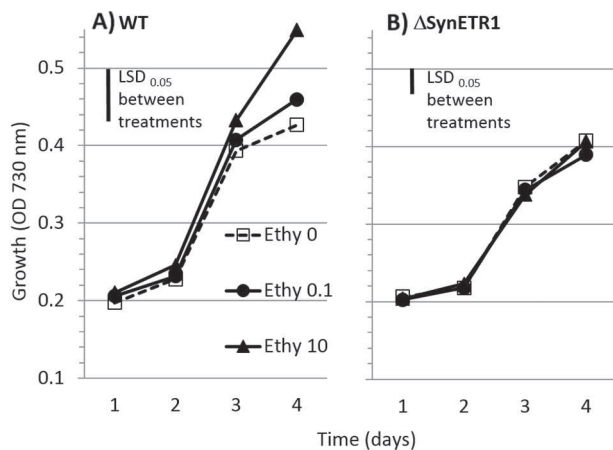


Fig. 2. Effects of various ethylene (“Ethy”) treatments on the *Synechocystis* growth measured by absorbance at 730 nm, A) in *Synechocystis* wild type and B) in *Synechocystis* Δ SynETR1, knock-out for the ethylene receptor. The various doses of ethylene “Ethy” (“0, 0.1, and 10”) are indicated in ppm. The points are means of 4 biological replicates. The LSDs (Least Significant Differences) were calculated at the 0.05 confidence level.

significant difference between 0.01 ppm and 0.1, 1 and 10 ppm. This stimulation reached > 10% at day 5, between 0.01 ppm and controls. The 100 ppm dose of ethylene tended to be less effective at stimulating growth with a + 5% in growth compared to controls. The exact ethylene doses can be checked in the supplemental data (Supp. Fig. 1).

Logically, the presence of 1-MCP significantly inhibited *Synechocystis* growth (Fig. 1). The MCP dose showing the strongest inhibition was 10 ppm, inducing a decrease by 10% compared to controls on the fifth day of culture. Thus it seems that ethylene perception is critical for normal *Synechocystis* growth.

To confirm that this is a specific effect of ethylene via a signaling pathway, we observed the effects of ethylene on the growth of the Δ SynETR1 strain with the ethylene receptor deleted. As shown in Fig. 2, the growth of the wild type strain is stimulated by ethylene, whereas, the growth of the Δ SynETR1 strain is not enhanced by any dose of ethylene. These experiments were run at different dates compared to those of Fig. 1, thus explaining the slightly different initial ODs and growth profiles.

3.2. Ethylene perception mediates *Synechocystis* photochemical efficiency of PSII

The enhancement of cyanobacterium growth in an autotrophic environment can be due to changes in its ability to convert light to energy. Indeed, Lacey and Binder [1], found that application of ethylene resulted in a 27% increase in the levels of PSII. Baker [21] summarized ways of measuring photosynthetic activity and Fv/Fm was proposed as an indicator of the potential photochemical efficiency of PSII in living organisms. Thus, we decided to measure Fv/Fm to estimate the potential photochemical efficiency of PSII in our experimental conditions. The results of Fig. 3, show ethylene-treated *Synechocystis* have significantly higher Fv/Fm values than controls and 1-MCP treated *Synechocystis* have a significantly lower Fv/Fm values than controls. Values of Fv/Fm were close to Fv/Fm ratio reported for *Synechocystis* cultures by Touloupakis et al. [16]. Fig. 3 shows the results of the Fv/Fm measurements taken at the 4th day of growth, during the log phase of growth. That is the stage at which the growth differences appeared (Fig. 1) and at which the Fv/Fm ratios were the highest (data not shown). Thus, it seems that ethylene perception is linked to optimal photosynthesis efficiency, supporting Lacey and Binder’s model for the role of ethylene [1]. In an additional experiment comparing wild type and Δ SynETR1 strains, we observed that ethylene had no significant effect on Fv/Fm in *Synechocystis* Δ SynETR1 strain (Supp. Fig. 2). Thus, ethylene is affecting photosynthesis efficiency via a specific signaling

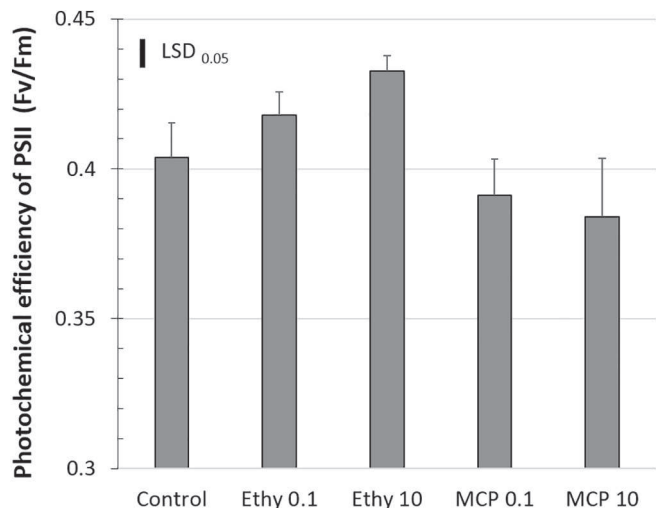


Fig. 3. Effect of ethylene or 1-MCP treatments on the potential photochemical efficiency of PSII (Fv/Fm) in wild type *Synechocystis*. Cell cultures were exposed (or not exposed in controls) to 0.1 ppm (Ethy 0.1) or 10 ppm (Ethy 10) of ethylene, or 0.1 ppm (MCP 0.1) or 10 ppm (MCP 10) of 1-MCP. Fv/Fm was measured at the 4th day of growth. Error bars stand for the standard error, $n = 6$. The LSD (Least Significant Difference) was calculated at the 0.05 confidence level.

pathway.

In cyanobacteria, changes in Fv/Fm under conditions of constant pigment content correlate well with changes in independent measurements of PSII function, but the absolute level of Fv/Fm is not a reliable indicator of the maximal photochemical efficiency of PSII [17]. A more complete analysis of different fluorescence parameters is needed to better appreciate the effect of ethylene on the photochemical efficiency of PSII. This also matches previous observations made in mutant tobacco impaired in ethylene perception [22]. These authors noticed that photosynthesis was affected, as they measured lower amounts in electron transport and Rubisco in ethylene perception mutants. Thus, a role for ethylene signaling in photosynthesis may be very ancient in evolution.

3.3. Ethylene changes the fatty acid content in wild type *Synechocystis*

As a recent study showed that ethylene can modulate the fatty acid profile in a green microalga, of the *Chlorella* genus [12], we checked the influence of exogenous ethylene and 1-MCP in *Synechocystis*. As shown in Fig. 4B, C and D, the levels of C16:0, C18:0 and C18:1 were enhanced by the addition of exogenous ethylene. In comparison to control levels, the increase was only significant with ethylene doses at 1 ppm and above (Fig. 4B, C, D), but the trend was clear: these three fatty acids increased with increasing ethylene doses. Significant increases of C16:0 and C18:0 were also observed with 1-MCP treatments (Fig. 4B and C). Finally, compared to control levels, the sum of the measured fatty acids increased by 22.5% after a 100 ppm ethylene treatment and by 15.5% after a 10 ppm 1-MCP treatment (Supp. Fig. 3). These fatty acid contents are consistent with previous studies in *Synechocystis* [23,24].

Our results suggest that ethylene and 1-MCP, modulate fatty acids contents during the growth phase of *Synechocystis*. A similar trend was observed recently in a different microalga, *Chlorella vulgaris* [12] with the use of ethephon, a compound releasing ethylene. However, this microalga has a different fatty acid profile, as the C18:3 is the most represented. The fact that 1-MCP acts in a similar way to ethylene on the fatty acid content is surprising. This needs further research to be deciphered. The relation between ethylene signaling and fatty acid content may be a new field of research. In plants or cyanobacteria, the regulation of fatty acid contents, at least C16 to C18, by ethylene has not been studied, to our knowledge. So further study will be necessary to better understand this trend.

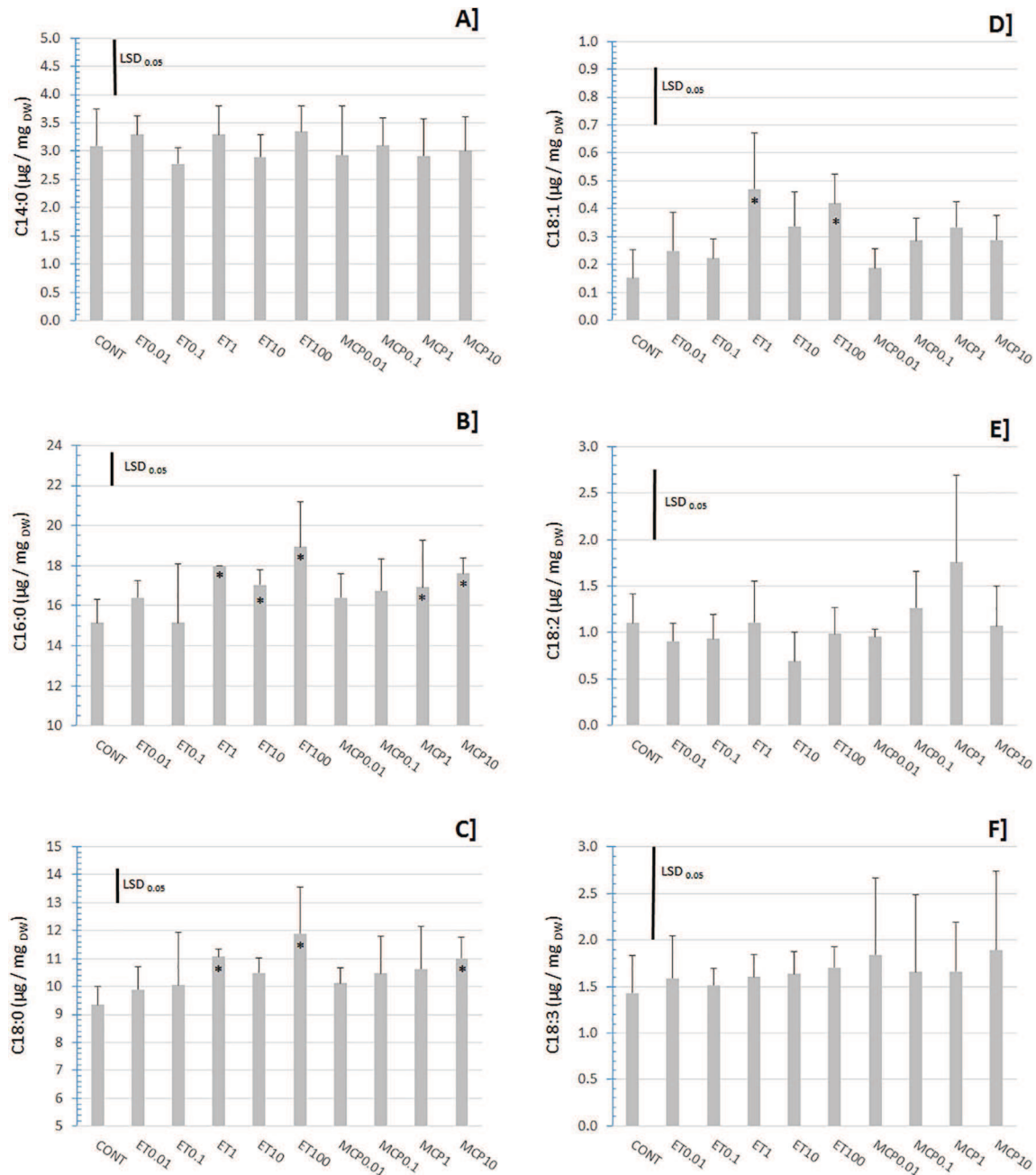


Fig. 4. Effects of ethylene (“ET”) and 1-methylcyclopropene (“MCP”) treatments on the fatty acid composition of *Synechocystis*. The ET and MCP doses are indicated in ppm. $n = 4$ biological replicates, the error bars stand for standard errors, the LSDs (Least Significant Differences) were calculated at the 0.05 confidence level. The asterisk (*) highlights a significant difference ($P < 0.05$) in comparison to the control (“CONT”) level.

3.4. Wild type *Synechocystis* mediates ethylene production

During the experiments described above we observed a very slight ethylene accumulation in control cells (Supp. Fig. 1 “set at 0 ppm”). Thus we ran a few additional experiments to check if the *Synechocystis* cells were able to produce ethylene. As shown in the Fig. 5, we observed a significant production of ethylene by the wild type *Synechocystis* incubated under darkness in the presence of methionine (“Methio. + cells”), compared to the ethylene production by the medium lacking cells (“Methio.”). However, a BLAST search within the *Synechocystis* PCC6803 whole protein list (http://genome.microbedb.jp/blast/blast_search/cyanobase/Synechocystis/genes) did not return any match when searching for the ACC synthase (ACS, the enzyme that produces ACC), nor when searching for ACC oxidase (ACO, the enzyme that

produces ethylene from ACC in plants) of *Arabidopsis thaliana*. So the classical plant route for synthesizing ethylene seems ruled out, and this was confirmed by incubating the culture with 1 mM AVG (aminoethoxyvinyl glycine, a known ACS inhibitor) that did not change ethylene production (data not shown). Another BLAST search within the *Synechocystis* PCC6803 proteins, gave no results when searching for oxoglutarate dioxygenase. This was another possible pathway for ethylene synthesis, existing in *Pseudomonas syringae* pv. Pisi, according to Chague et al. [9]. These authors listed a third pathway for ethylene synthesis, which is via the α -keto γ -methylthiobutyric acid (KMBA). This compound is excreted, then photo-oxidized and ethylene is produced. This seems the most probable pathway. Indeed, under light conditions, there was more ethylene accumulation in the presence of cells or in medium that contained cells before the beginning of the

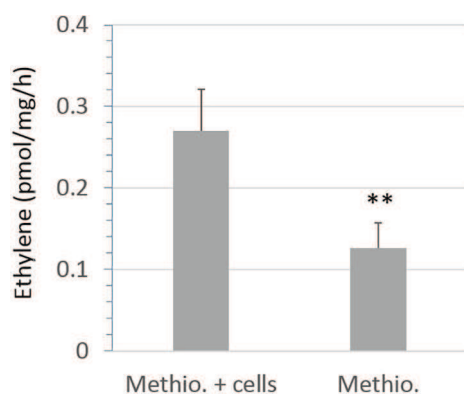


Fig. 5. Stimulation of ethylene accumulation by wild type *Synechocystis* PCC 6803 incubated under darkness in the presence of methionine "Methio" (10 mM). "cells" stands for the presence of *Synechocystis* cells, $n = 6$ biological replicates, error bars stand for standard errors, and ** shows a significant difference at the 0.01 level (t -test).

incubation with methionine (Supp. Fig. 4). As shown in this Supp. Fig. 4, other conditions were more variable. In light, addition of methionine may have led to an increase in ethylene production, but this was seen in the presence and absence of cells. In the dark, the low levels of ethylene production observed with methionine in cell-free conditions ("M") was comparable to the levels seen with cells in the absence of methionine ("C") and in the absence of both methionine and cells ("-"). Thus, addition of methionine to cells results in a measurable increase in ethylene levels. Moreover, we had inconsistent results with 2,4-dinitrophenylhydrazine, trying to reveal the KMBA presence as described in Chague et al. [9]. Finally, we also noticed that the basic pH of the medium was favorable to ethylene accumulation when adding methionine (data not shown). So more work is necessary to validate the KMBA hypothesis.

4. Conclusion

To our knowledge, this is the first report showing that exogenous ethylene enhances cyanobacterial growth, and 1-MCP, a specific inhibitor of ethylene receptors, inhibits cyanobacterial growth. This was validated by the absence of an ethylene effect on the growth of a *Synechocystis* strain lacking the ethylene receptor. In accordance to these observations, we also observed that ethylene and 1-MCP modulate the photochemical efficiency of PSII of the cyanobacterium, as suggested by Lacey and Binder [1] using different measurements. The SynEtr1 gene had already been described and called Positive phototaxisA, *pixA* [25]. Additionally, we observed that the ethylene modulates the fatty acid accumulation, leading to higher amounts of C16:0, C18:0 and C18:1. Modulation of fatty acids by an ethylene precursor, ethephon, has already been observed in *Chlorella vulgaris* under different conditions [12].

Finally, we observed a slight increase in ethylene production by wild type *Synechocystis* PCC 6803 in the presence of methionine, but we are unsure about the biosynthetic pathway involved in this. It has been previously observed that ethylene production by wild type *Synechocystis* PCC 6803 was light dependent [26]. However, these earlier results used ACC, the immediate precursor of ethylene in plants. We found that ACC in cell-free BG11 was converted to ethylene (data not shown) calling into question these earlier results. A more recent study observed no ethylene production in the wild type strain [27], but the sensitivity of the detection method was low (nmol/mg/h). Thus, further studies are necessary to determine the biosynthesis pathway and conditions where ethylene biosynthesis occurs in *Synechocystis*.

Author's contributions

MA, PYP, CVG, BB and CC conceived and designed the study; MLH,

MC, AL, IP, PM and CC acquired the data; MLH, MC, MA, PM and CC analyzed and interpreted the data; MLH, MA, MC, PM, AL, IP, BB and CC draft the article; BB, CVG, PYP and CC revised it critically for important intellectual content. Finally, all co-authors approved the final version to be submitted. Additional contributions were statistical expertise by CVG and obtaining of funding by PYP and CC.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jgal.2017.07.032>.

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