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Enhancement of thiamine release during synthetic mutualism between *Chlorella sorokiniana* and *Azospirillum brasilense* growing under stress conditions

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Abstract Thiamine release during synthetic mutualism between *Chlorella sorokiniana* co-immobilized in alginate beads with the microalgae growth-promoting bacterium *Azospirillum brasilense* was measured under stress conditions of pH, light intensity, and nitrogen starvation in short-term experiments. Thiamine release in the co-immobilized treatment was significantly higher at acidic pH compared to thiamine released by either microorganism alone. Under slightly alkaline pH, *C. sorokiniana* released the highest amount of thiamine. At stressful pH 6, the co-immobilized treatment released a higher quantity of thiamine than the sum of thiamine released by either microorganisms when immobilized separately. Release of thiamine by *C. sorokiniana* alone or co-immobilized was light intensity dependent; with higher the light intensity, more thiamine was released. Extreme light intensity negatively affected growth of the microalgae and release of thiamine. Nitrogen starvation during the first 24 h of culturing negatively affected release of thiamine by both microorganisms, where *C. sorokiniana* was more severely affected. Partial or continuous nitrogen starvation had similar negative effects on *C. sorokiniana*, but co-immobilization improved thiamine release. These results indicate that thiamine is released during synthetic mutualism between *C. sorokiniana* and *A. brasilense*, and this happens specifically during the alleviation of pH stress in the microalgae.

Keywords *Azospirillum* · Alleviation of stress · Microalgae · Plant growth-promoting bacteria

Introduction

Mutualistic interactions, either naturally occurring or man-made (synthetic mutualism—Imase et al. 2008; Momeni et al. 2011), among different species of either microorganisms or macroorganisms can improve the performance of the partners and are based on the exchange of resources and services (Bronstein 1994; Doebeli and Knowlton 1998; Kessler and Heil 2011). The composition of exudates produced by an organism can indicate the type of microorganisms that are interacting in a specific environment (Kamilova et al. 2006). Exudates from plant are a key determinant of the microbial community structure in the rhizosphere (Barea et al. 2013; Cesco et al. 2012; Nannipieri et al. 2008; Hartmann et al. 2009).

Vitamins are one of the lesser-studied compounds in plants and microalgae exudates, and most studies are decades old (Aaronson et al. 1977; Nishijima et al. 1979; Schönwitz and Ziegler 1994). Green unicellular microalgae of the genus

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This study is dedicated to the memory of the German/Spanish mycorrhizae researcher Dr. Horst Vierheilig (1964–2011) of CSIC, Spain

The term "release" stands for "extrusion", "excretion", and "produce". A specific term is used when the exact mechanism is known.

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Chlorella produce and exude several vitamins, such as thiamine, riboflavin, biotin, cobalamin, and pantothenic acid (Aaronson et al. 1977; Nishijima et al. 1979; Pratt and Johnson 1965). These microalgae are intensively studied for many biotechnological applications (Lebeau and Robert 2006; de-Bashan and Bashan 2010; Perez-Garcia and Bashan 2015).

Diverse plant growth-promoting bacteria (PGPB) can produce vitamins in artificial culture media, as well. At least 11 vitamins are produced by PGPB in vitro and, so far, seven of these occur in relation to interactions with plants. Growth parameters of in vitro culturing play the major role determining which vitamin is produced and in what quantity (Palacios et al. 2014). Specifically, this study used the PGPB *Azospirillum brasilense.* The general characteristics of this PGPB is a highly motile bacterium (Bashan and Holguin 1994; Bashan and Levanony 1987), having high affinity for colonizing roots (Bashan et al. 1986; Levanony et al. 1989; Puente et al. 1999), but poor survival in soils without plants (Bashan et al. 1995; Bashan 1999). It affects plant growth, including microalgae, by many small-scale mechanisms working together or in cascade, a theory called “multiple mechanism theory” (Bashan and de-Bashan 2010). This PGPB can produce several vitamins in vitro, including thiamine, riboflavin, biotin, pantothenic acid, and niacin (Dahm et al. 1993; Rodelas et al. 1993). Production mainly depends on the kind of carbon source in the culture media and on environmental factors, such as temperature and pH (Dahm et al. 1993; Rodelas et al. 1993).

Functions and requirements for vitamins in plants and bacteria have been extensively studied (Smith et al. 2007 and references therein). Specifically, thiamine serves as a co-factor in diverse metabolic pathways in plants (Schyns et al. 2005). During plant–microbe interactions, vitamins influence proliferation of PGPB in and around the root system, thereby enhancing their competitiveness (Palacios et al. 2014). Because of the common occurrence of microbial vitamin production by PGPB and assimilation of microbe-synthesized vitamins by plants, vitamins have a role in plant development and rhizosphere interactions (Baya et al. 1981).

The main factors affecting production of vitamins in microalgae and bacteria are environmental conditions, especially temperature, pH, nutrient deficiencies, and light intensity. Some changes from optimal conditions produce stress in microorganism. The pH of the culture medium is one of the most important factors, which affects optimal growth of algal cultures (Khalil et al. 2010). Intense light has a strong negative effect on pigment concentration (Kana et al. 1997). Limited available nitrogen or nitrogen starvation affects microalgae in different ways, particularly carbohydrate and lipid metabolism in general and specifically in *Chlorella* spp. (Choix et al. 2012a, b; Khozin-Goldberg and Cohen 2011; Přibyl et al. 2012; Widjaja et al. 2009).

Many PGPB and especially *Azospirillum* spp. can mitigate diverse stressors, including salt, osmotic, and oxidative stress in plants (Bacilio et al. 2004; Bashan and de-Bashan 2010; Cassán et al. 2009; Rodrigues et al. 2013; Rojas-Tapias et al. 2012). In the microalgae *Chlorella sorokiniana* UTEX 2714 (formerly *C. vulgaris*), when co-immobilized with the PGPB *A. brasilense*, this can ameliorate pH and tryptophan stress (de-Bashan et al. 2005), promote cellular and population growth (Gonzalez and Bashan 2000), and enhance production of carbohydrates, lipids, fatty acids, and photosynthetic pigments (Choix et al. 2012a, b; de-Bashan et al. 2002; Leyva et al. 2014). Consequently, *Chlorella* spp. and *A. brasilense* were proposed as a model to study plant–bacteria interactions (de-Bashan and Bashan 2008). This created a synthetic mutualism where numerous positive effects were recorded for both partners. So far, the microalgae have received more attention (de-Bashan et al. 2015).

Thiamine is required by all cells because of its role as a co-factor in the formation of thiamine pyrophosphate for enzymes essential to carbon and amino acid metabolism (Jurgenson et al. 2009). The specific role of thiamine during plant–bacteria interaction, as a co-factor in different metabolic pathways, activates defense reactions in plants and promotes plant growth (for review see Palacios et al. 2014). To start to explain the possible roles of thiamine during mutuality interaction of the microorganisms, it is essential to explore the conditions that are conducive for this vitamin under mutual culturing. To this end, we studied the release of thiamine during artificial mutualism between *C. sorokiniana* and *A. brasilense.* We specifically tested the hypothesis that cultivating *A. brasilense* with *C. sorokiniana* in very close proximity in an alginate bead affects the release of thiamine by the bacteria–microalgae association under stressors of low and high light intensities, low and high pH, and presence or absence of a nitrogen source, compared to ideal culturing. Microorganisms immobilized alone served as controls.

Materials and methods

Microorganisms and initial growth conditions prior experiments

The unicellular microalgae *Chlorella sorokiniana* (UTEX 2714, University of Texas, Austin, TX) (formerly *Chlorella vulgaris* Beijerink; Bashan et al. 2015) and the bacterium *Azospirillum brasilense* Cd (DSM 1843; Leibniz-Institut DMSZ, Braunschweig, Germany) were used. To produce cultures of the microalgae, 10 mL of axenic culture of microalgae was inoculated into 90 mL sterile mineral medium (C30), as described in Gonzalez et al. (1997). The medium contains (in g L^{-1}) KNO_3 (25), $MgSO_4{\cdot}7H_2O$ (10), KH_2PO_4 (4), K_2HPO_4 (1), and $FeSO_4{\cdot}7H_2O$ (1) and contains (in μg L^{-1}) H_3BO_3

(2.86), $MnCl_2 \cdot 4H_2O$ (1.81), $ZnSO_4 \cdot 7H_2O$ (0.11), $CuSO_4 \cdot 5H_2O$ (0.09), and $NaMoO_4$ (0.021). The inoculated medium was incubated at 27±2 °C at 140 rpm under light intensity of 60 μmol photons m^{-2} s^{-1} for 7 days, then harvested by centrifugation at 6000×*g* for 5 min. To eliminate traces of growth medium, the pellets were rinsed three times in saline solution (0.85 % NaCl *w/v*). The bacterium was cultivated in BTB-2 medium (Bashan et al. 2011), which contains (in g L^{-1}) NaCl (1.2), $MgSO_4 \cdot 7H_2O$ (0.25), K_2HPO_4 (0.13), $CaCl_2$ (0.22), K_2SO_4 (0.17), NH_4Cl (1), Na_2SO_4 (2.4), $NaHCO_3$ (0.5), Na_2CO_3 (0.09), Fe-EDTA (0.07), tryptone (5), and yeast extract (5), with 8 mL glycerol. The pH was adjusted to 7 with 1 M KOH, incubated at 32±2 °C at 120 rpm for 16 h, and then harvested by centrifugation at 6000×*g* for 5 min. Elimination of traces of growth medium was done as described earlier.

Immobilization of microorganisms

The microorganisms were immobilized, using the method described by de-Bashan et al. (2004), where 40 mL *C. sorokiniana* culture (6.0×10^6 cells mL^{-1}) were mixed with 160 mL sterile, 6000 cP 2 % alginate solution (alginate mixed at 14,000 and 3500 cP), and stirred for 15 min. Using an automatic bead maker, this mixture was dropped into a 2 % $CaCl_2$ solution under slow stirring (de-Bashan and Bashan 2010). The beads were stabilized for 1 h at 28±1 °C and washed in sterile saline solution (0.85 % NaCl). *Azospirillum brasilense* (approximately 1.0×10^9 CFU mL^{-1}) was immobilized similarly. Immobilization normally reduces the number of *A. brasilense* but not *C. sorokiniana* in the beads; therefore, a second incubation step for recovering the lost population of the bacteria (secondary growth) was necessary (nutrient broth, 10 %, *w/v*, #N7519 Fluka-Sigma-Aldrich) (Bashan 1986). Under starvation conditions for secondary multiplication, we used N-free OAB medium containing (in g L^{-1}) KOH (4.80), malic acid (5), NaCl (1.20), $MgSO_4 \cdot 7H_2O$ (0.25), K_2HPO_4 (0.13), $CaCl_2$ (0.22), K_2SO_4 (0.17), Na_2SO_4 (2.40), $NaHCO_3$ (0.50), Na_2CO_3 (0.09), and $Fe_{III}EDTA$ (0.07), and (in μg L^{-1}) H_3BO_3 (0.2), $MnCl_2 \cdot 4H_2O$ (0.2), $ZnCl_2$ (0.15), $CuCl_2 \cdot 2H_2O$ (0.2), and $NaMoO_4 \cdot 2H_2O$ (20) (Bashan and de-Bashan 2015). Light intensity, temperature, and pH in the secondary multiplication of the bacterium were similar to ideal conditions of growth under experiments described earlier. For co-immobilizing the two microorganisms in the same bead, after washing the cultures, each culture was re-suspended three times in 10 mL sterile saline solution (0.85 % NaCl *v/v*) and then mixed in the alginate before forming the beads. It is well established that immobilization of these microorganisms in alginate beads prevents their release from the beads (Covarrubias et al. 2012). Under close confinement, they are forced to interact with each other (de-Bashan and Bashan 2008; de-Bashan et al. 2015).

Experimental culture conditions

After secondary incubation, the beads were washed three times in sterile saline solution to remove remnants of the medium. For each experiment, 20 g of beads with microorganisms, either immobilized alone or co-immobilized, was inoculated in 150 mL synthetic growth medium (SGM), described in de-Bashan et al. (2011), containing (in mg L^{-1}) NaCl (7), $CaCl_2$ (4), $MgSO_4 \cdot 7H_2O$ (2), K_2HPO_4 (217), KH_2PO_4 (8.5), Na_2HPO_4 (33.4), and NH_4Cl (191). The inoculated medium was incubated under autotrophic conditions under different light intensities, pH, and nitrogen conditions: (1) four different light intensities (30, 60, 300, and 500 μmol photons m^{-2} s^{-1}) at pH 7; (2) pH 6 (adjusted with 1 N HCl) and pH 7 and pH 8 (adjusted with 0.3 N NaOH) at 60 μmol photons m^{-2} s^{-1}. The N starvation experiments were conducted in synthetic N-free-medium at pH 7, 60 μmol photons m^{-2} s^{-1}. (3) Two regimens of nitrogen starvation were tested. The first was done during secondary growth (24 h after immobilizing the microorganisms) and a second regimen during the entire experiment. The diazotroph *A. brasilense* does not fix atmospheric nitrogen during highly oxygenated culturing conditions with *C. sorokiniana*, maintaining the N-free medium. All experiments were stirred at 140 rpm at 27±2 °C for 144 h.

Cell counting of microorganisms

For each treatment, three beads from a 250-mL flask were sampled. Each bead was solubilized by immersion for ~30 min (~28 °C) in 1 mL of citrate buffer containing (in mM) sodium citrate (55), EDTA anhydride (30), and $NaCl_2$ (150), in a final volume of 1 L and adjusted to pH 8 with NaOH. *Azospirillum brasilense* cells were counted by the fluorescein diacetate method described by Chrzanowski et al. (1984) under a fluorescent microscope (BX41, Olympus, Japan). *Chlorella sorokiniana* was counted under a light microscope using a Neubauer hemocytometer connected to an image analyzer (Image ProPlus 4.5; Media Cybernetics, USA) (Gonzalez and Bashan 2000).

High-performance liquid chromatography (HPLC) analysis

HPLC (1100, Agilent Technologies, USA) was used for all analyses. Chromatograms were analyzed and recorded using HPCHEM integrating software (version G2170BA; Agilent Technologies). A modification of a HPLC method by Sánchez-Machado et al. (2004) was used. The HPLC system was equipped with a reversed phase column (LiChrosorb-RP 18, 10 μm particle size, 250×4 mm; Merck, Germany) and was run isocratically (all analyses use the same concentration of solvents) using 0.1 M phosphate buffer (pH 7)/acetonitrile (72:28 *v/v*) serving as the mobile phase. The injection volume was 30 μL and the flow rate was 0.65 mL min^{-1}. Thiamine and its derivatives, thiamine monophosphate and thiamine diphosphate, were measured indirectly by fluorescence detection after post-oxidation to thiochrome, thiochrome monophosphate, and thiochrome diphosphate, respectively. The post-oxidation procedure was 500 μL 1 % potassium ferricyanide (dissolved in 15 % aqueous NaOH) added to 1 mL standard solution or sample; the solution was shaken for 10 s and then mixed with 100 μL concentrated H_3PO_4. The settings of the fluorescence detector were 370 nm for excitation and 435 nm for emission. For vitamin B_1 and its two derivatives, the standards were thiamine hydrochloride (T-4625; Sigma), thiamine monophosphate chloride dehydrate (T-8637; Sigma), and thiamine diphosphate (C-8754; Sigma).

Determination of thiamine in exudates

One gram of beads (approximately 25 beads per replicate and per treatment) was dissolved in 15 mL citrate buffer as described above. Once dissolved, sample was centrifuged at 6000×*g* for 5 min. The pellet was discarded and the supernatant was added to 10 mL of the culture medium to obtain at the end a mixed sample (25 mL) of exudates representing both inside and outside beads. The solutions were centrifuged at 6000×*g* for 10 min at room temperature, filtered through a 0.22-μm membrane filter (GSWP02500; EMD Millipore), and analyzed by HPLC. Net release of thiamine was measured by subtracting the thiamine released by each microorganism immobilized alone from the amount of thiamine released by the co-immobilized treatment.

Experimental designs and data analysis

The setup of all experiments was in batch cultures. Each experiment was performed in triplicate, where each 250-mL Erlenmeyer flask served as a replicate. Each setup contained three treatments: beads containing *C. sorokiniana*, beads containing *A. brasilense* (these two serving as controls), and beads containing the two co-immobilized microorganisms (n=9). Each experiment was repeated twice. The quantity of thiamine made of the sum of thiamine monophosphate and thiamine diphosphate are expressed as total thiamine per milliliter of the solution. The linearity of the calibration curves was tested by a correlation analysis, followed by a regression analysis. Data of counted cells and vitamin production were analyzed first by one-way ANOVA and then by LSD (least statistical difference) post hoc analysis, with significance set at $P<0.05$, using statistical software (Statistica 6.0; StatSoft, USA). The comparison between thiamine production by co-immobilized treatment versus thiamine production by *C. sorokiniana* and *A. brasilense* (immobilized separately) were analyzed by orthogonal contrast analysis with significance set at $P<0.05$, using analytical software (SAS 9.00; SAS Institute, USA).

Results

Effect of pH of the growth medium on thiamine release

Thiamine released during co-immobilization of *C. sorokiniana* with *A. brasilense* was significantly higher at pH 6 after 96 h compared to thiamine released by each microorganism immobilized alone (Fig. 1a). The amount of thiamine released by co-immobilized microorganisms (116±45.3 ng mL^{-1}) was higher than the sum of the thiamine concentration released by either microorganism immobilized alone (28.5±8.2 ng mL^{-1} for *C. sorokiniana*; 37.3±4 ng mL^{-1} for *A. brasilense*; $P<0.0045$). A similar result was obtained at pH 7, where exudation of thiamine in the co-immobilized treatment (64.5±4.02 ng mL^{-1} after 96 h) was also higher than the amount released by either of the microorganisms immobilized alone (Fig. 1b). At pH 8, *C. sorokiniana* released the highest amount of thiamine after 96 h (241.7±144.7 ng mL^{-1}) (Fig. 1c). At other sampling times, the co-immobilized treatment released amounts of thiamine that were similar to the sum of thiamine released by each microorganism immobilized alone. These quantities were lower than the quantities released at pH 6. Analyzing the same data and comparing the effects of different pH treatments revealed that pH had no significant effect on release of thiamine by either microorganism (except at pH 8 for *C. sorokiniana* and pH 6 and 7 for co-immobilized microorganisms after 96 h) (Fig. S1a–c). At pH 6 and 7, a gain in thiamine was detected, with a net release of thiamine by the co-immobilized microorganisms of 50.19±20.97 ng mL^{-1} at pH 6 and 9.2856±1.0125 ng mL^{-1} at pH 7. At pH 8, the yield of thiamine in the co-immobilized treatment was similar to the sum of the thiamine released by *C. sorokiniana* and *A. brasilense* immobilized alone, indicating no gain of thiamine (Fig. 1c).

Regardless of the pH, *C. sorokiniana* had larger populations when co-immobilized with *A. brasilense* (Fig. 1d–f, Fig. S1d, f). When immobilized alone, cultures of *C. sorokiniana* grown at pH 6 had significantly smaller populations (Fig. 1d). However, when co-immobilized, the population recovered and no significant difference was detected between populations growing at different pH levels (Fig. S1f); this indicates alleviation of stress. At all pH levels of the cultures of *A. brasilense*, populations were always smaller in the co-immobilized treatment (Fig. 1g–i, Fig. S1e).

Effect of light intensity on thiamine release

Under all light conditions, both microorganisms released thiamine into the growth medium (Fig. 2), and this release was light intensity dependent in the range of 30–

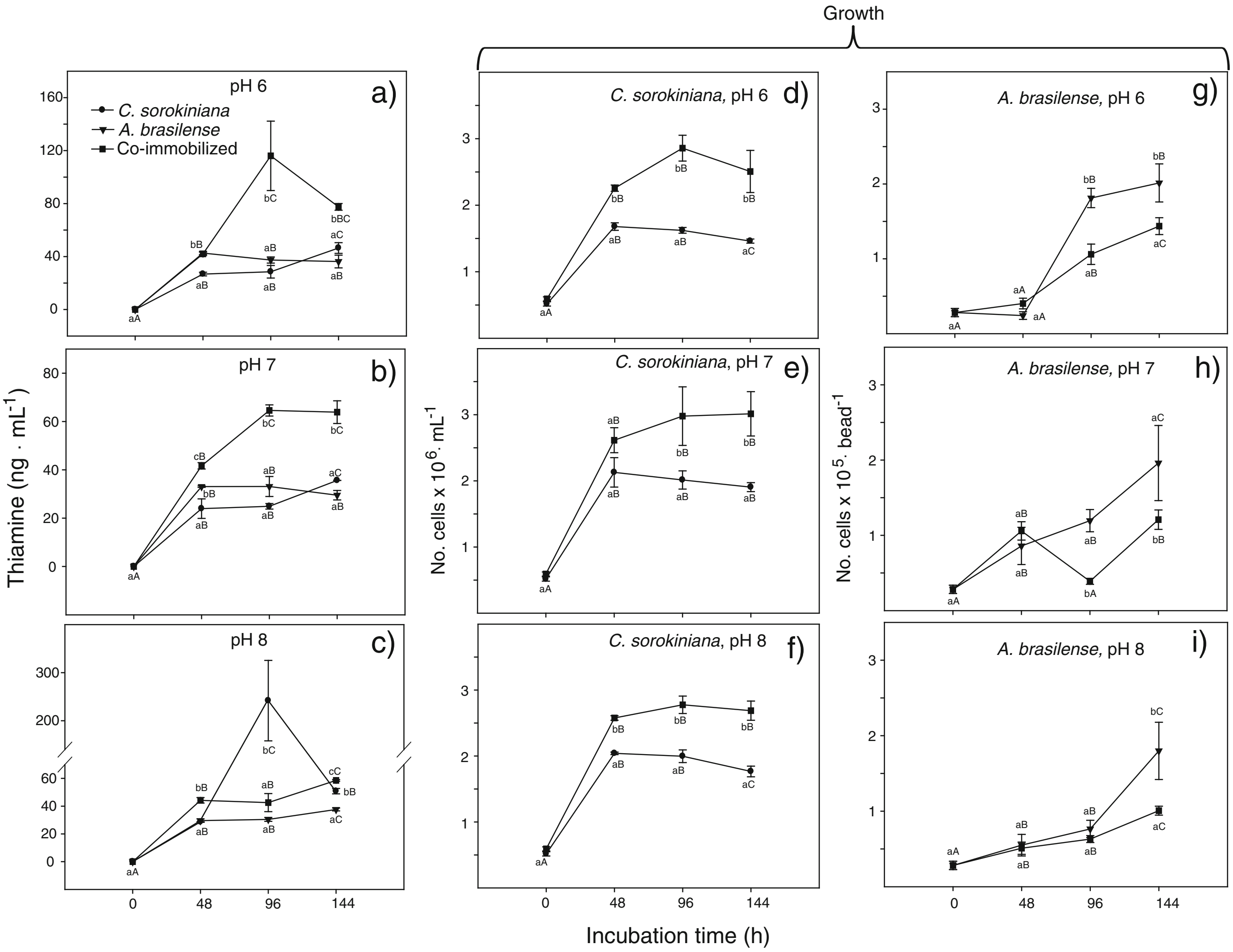

Fig. 1 Thiamine in exudates at pH 6 (**a**), 7 (**b**), and 8 (**c**) produced and released by *Chlorella sorokiniana* and *Azospirillum brasilense* immobilized separately or immobilized together in alginate beads and growth of these microorganisms in these cultures (**d**, **e**, **f**, **g**, **h**, **i**). Values along curves denoted by *different capital letters* differ significantly, using one-way ANOVA and LSD post hoc analysis at $P < 0.05$. Points at each time interval denoted by different *lower case letters* differ significantly at $P < 0.05$ by the same statistical analyses. *Bars* represent SE

300 μmol photons m^{-2} s^{-1}. As light intensity increased, thiamine release increased (Fig. 2a–c, Fig. S2a), reaching the highest level at 300 μmol photons m^{-2} s^{-1}. At 500 μmol photons m^{-2} s^{-1}, release of thiamine dropped to the lowest level of all treatments (Fig. 2d, Fig. S2a–c).

At 30 μmol photons m^{-2} s^{-1}, there is no difference in the amount of thiamine released by either treatment. At 60 and 500 μmol photons m^{-2} s^{-1} at 96 h, more thiamine was released in the co-immobilized treatments; however, only at 60 μmol photons m^{-2} s^{-1} was more thiamine released by the co-immobilized than the sum of thiamine released by each microorganism immobilized alone (Fig. 2b). At this time duration and light intensity, the net release of thiamine in the co-immobilized treatment was 6.22±3.01 ng mL^{-1}. At 300 μmol photons m^{-2} s^{-1}, the highest release of thiamine occurred in *C. sorokiniana* alone and co-immobilized; only after 144 h is there a difference between the two (Fig. 2c, Fig. S2a, c). At this time duration and light intensity, net release of thiamine in the co-immobilized treatment was 6.22±3.01 ng mL^{-1}.

Except under 30 μmol photons m^{-2} s^{-1}, *A. brasilense* enhanced growth of *C. sorokiniana* in all cases (Fig. 2e–h). Although there was no growth of *C. sorokiniana* at 500 μmol photons m^{-2} s^{-1}, in co-immobilization with *A. brasilense* after incubation for 144 h was there a slight, but significant, enhanced growth (Fig. 2h), indicating a reduction of stress after an adaptation period. Growth of *A. brasilense* was similar at all the tested light intensities (Fig. S2e).

Effect of nitrogen starvation on release of thiamine

Release of thiamine by *C. sorokiniana*, when immobilized alone, ceased after 48 h of incubation (8.81±3.23 ng mL^{-1}).

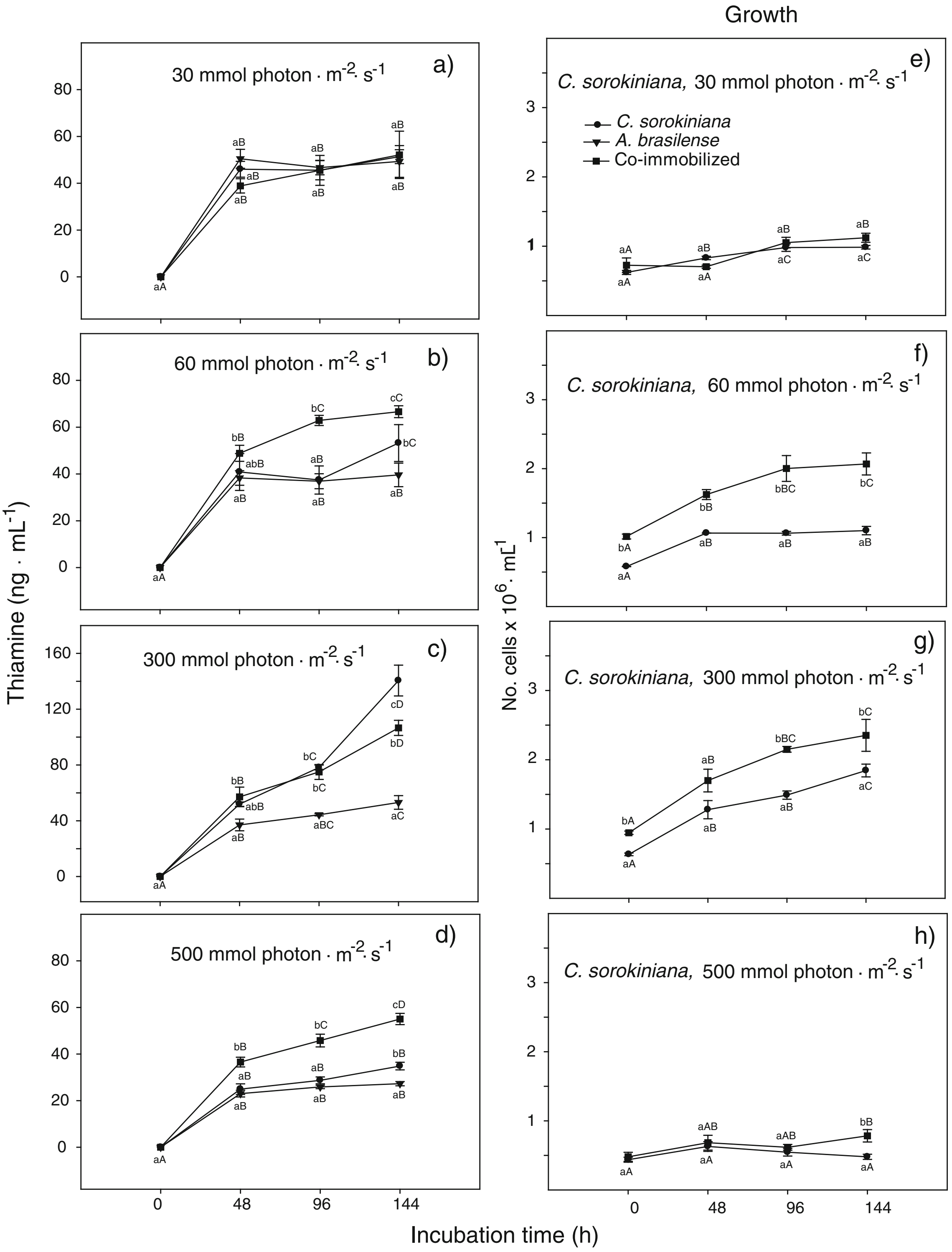

Fig. 2 Comparison of thiamine content in exudates under light intensities of 30, 60, 300, and 500 μmol photon m^{-2} s^{-1} released by *Chlorella sorokiniana* and *Azospirillum brasilense* immobilized separately or immobilized together in alginate beads (**a**, **b**, **c**, **d**) and growth of *Chlorella sorokiniana* immobilized alone and co-immobilized with *Azospirillum brasilense* in these cultures (**e**, **f**, **g**, **h**). Values along curves denoted by different *capital letters* differ significantly using one-way ANOVA and LSD post hoc analysis at $P < 0.05$. Points at each time interval denoted by different *lower case letters* differ significantly at $P < 0.05$ by the same statistical analyses. *Bars* represent SE

Even when nitrogen was restored after 24 h of incubation, release of thiamine by *C. sorokiniana* did not recover (Fig. 3a, b). Nitrogen starvation during the first 24 h of the secondary growth phase negatively affected thiamine release by the microalga and the bacterium, but *C. sorokiniana* was more affected (Fig. 3b).

Release of thiamine under the co-immobilized treatment and in *A. brasilense* immobilized alone was similar under both conditions, whether the nitrogen was restored or not (Fig. 3a, b). The quantities of released thiamine in these treatments were at all sampling times higher than when *C. sorokiniana* was cultured alone (Fig. 3a, b). The sole difference was that during the co-immobilized treatment, release of thiamine slowly continued over the entire 144 h of the experiment (Fig. 3a, b). There was no significant difference in the growth of *C. sorokiniana*, whether immobilized alone or with *A. brasilense* in both nitrogen-absent regimes (Fig. 3c, d). However, under partial nitrogen starvation, *C. sorokiniana*, when immobilized alone or co-immobilized with *A. brasilense* and *A. brasilense* immobilized alone, growth was greater than under full starvation regimes (Fig. 3c–f).

Discussion

Synthetic mutualism between *C. sorokiniana* and *A. brasilense* is man-made and so far not found in nature. As is, it does not have ecological importance. This created mutualism has two purposes: (1) to study the metabolism of plant–bacterial interaction because it highly resembles, in several metabolic pathways, the interaction between PGPB and higher plants (de-Bashan and Bashan 2008; de-Bashan et al. 2015); and (2) to explore potential usefulness of this mutualism for biotechnological gains (see "Introduction" for references). Specifically, this study of thiamine extrusion, known to participate in PGPB–plant interactions (Palacios et al. 2014), and as a co-factor in carbon and amino acid metabolism in all organisms (Jurgenson et al. 2009), is aimed to describe new functional interactions and to increase our understanding of eukaryote–prokaryote cell interactions. At the same time, this research aims to provide a tool for biotechnological development. These ideas are supported by two examples of thiamine contribution to auxotrophic–thiamine marine microalgae: (1) co-culturing of the phytoplankton microalgae *Ostreococcus lucimarinus* with a *Pseudoalteromonas* sp., strain TW7. This strain, which synthesizes thiamine, showed the bacterial influence on thiamine availability, allowing the microalgae to grow. This bacterium can increase thiamine availability beyond de novo synthesis (Paerl et al. 2015). (2) The marine bacterium *Dinoroseobacter shibae*, a symbiont of cosmopolitan marine microalgae, can provide thiamine to auxotroph–thiamine microalgae (Wagner-Döbler et al. 2010). However, the novel contribution of this study is to show changes in available thiamine between two interacting microorganisms that can produce and consume thiamine and both are benefited from the interaction.

During synthetic mutualism, the PGPB *A. brasilense* enhanced production of diverse compounds, such as carbohydrates, lipids, and photosynthetic pigments in *C. sorokiniana* during their interaction within alginate beads (Choix et al. 2012a, b; Gonzalez and Bashan 2000; de-Bashan et al. 2002; Leyva et al. 2014). This study specifically extends the effects when both microorganisms interact under optimal or stressful growth conditions by releasing thiamine. This study demonstrated that, during synthetic mutualism, *C. sorokiniana*, co-immobilized with *A. brasilense*, enhances the release of thiamine into the growth medium under stress conditions of pH.

By measuring the relative effect of each stressor, only at pH 6 (stressful condition for growth of microalgae) more thiamine was released in the co-immobilized treatment than the sum of thiamine released by *C. sorokiniana* and *A. brasilense* when immobilized separately. This may happen because production of thiamine by microalgae is related to cell multiplication (Nishijima et al. 1979) and *A. brasilense* alleviates the effect of pH stress in *C. sorokiniana* (de-Bashan et al. 2005), leading to recovering and enhancement of growth in the microalga. Under non-stressed growth conditions at pH 7 and light intensity of 60 μmol photon m^{-2} s^{-1} (serving as controls in our study), more thiamine was released under the co-immobilized treatment. Under these optimal growth conditions, several metabolic effects have been reported for the microalgae *C. sorokiniana*, when co-immobilized with the bacteria *A. brasilense* (Gonzalez and Bashan 2000; Choix et al. 2012a, b, 2014; de-Bashan et al. 2002; Leyva et al. 2014, 2015). The well-established role of thiamine as a cofactor in several metabolic pathways (Schyns et al. 2005) can explain the enhancement of thiamine released to the media during optimal synthetic mutualism of these microorganisms, allowing more available thiamine for both species.

Release of thiamine and cell growth of *C. sorokiniana* was light dependent when immobilized alone or co-immobilized with *A. brasilense*. Lv et al. (2010) report that cell growth of *C. sorokiniana* increases with the increase of light intensity. Thiamine diphosphate acts as a co-factor of an important number of enzymes in carbohydrate metabolism (Schyns et al. 2005). Therefore, we theorized that the increase in carbon metabolism influenced by light intensity could induce greater production of thiamine (which also is able to be released to the medium) by *C. sorokiniana* that, in turn, facilitates carbon assimilation. Yet, light may have limits. We found that under a high light intensity, release of thiamine and population growth of *C. sorokiniana*, when immobilized alone, were negatively affected. It is plausible that the effect of the highest light intensity on growth of *C. sorokiniana* is due to a photoinhibition, a light-induced depression of photosynthesis caused by excessive light (Smith and Underwood 2000). Similar effects have been reported for other microalgae (Kana et al. 1997; Pal et al. 2011). When *C. sorokiniana*

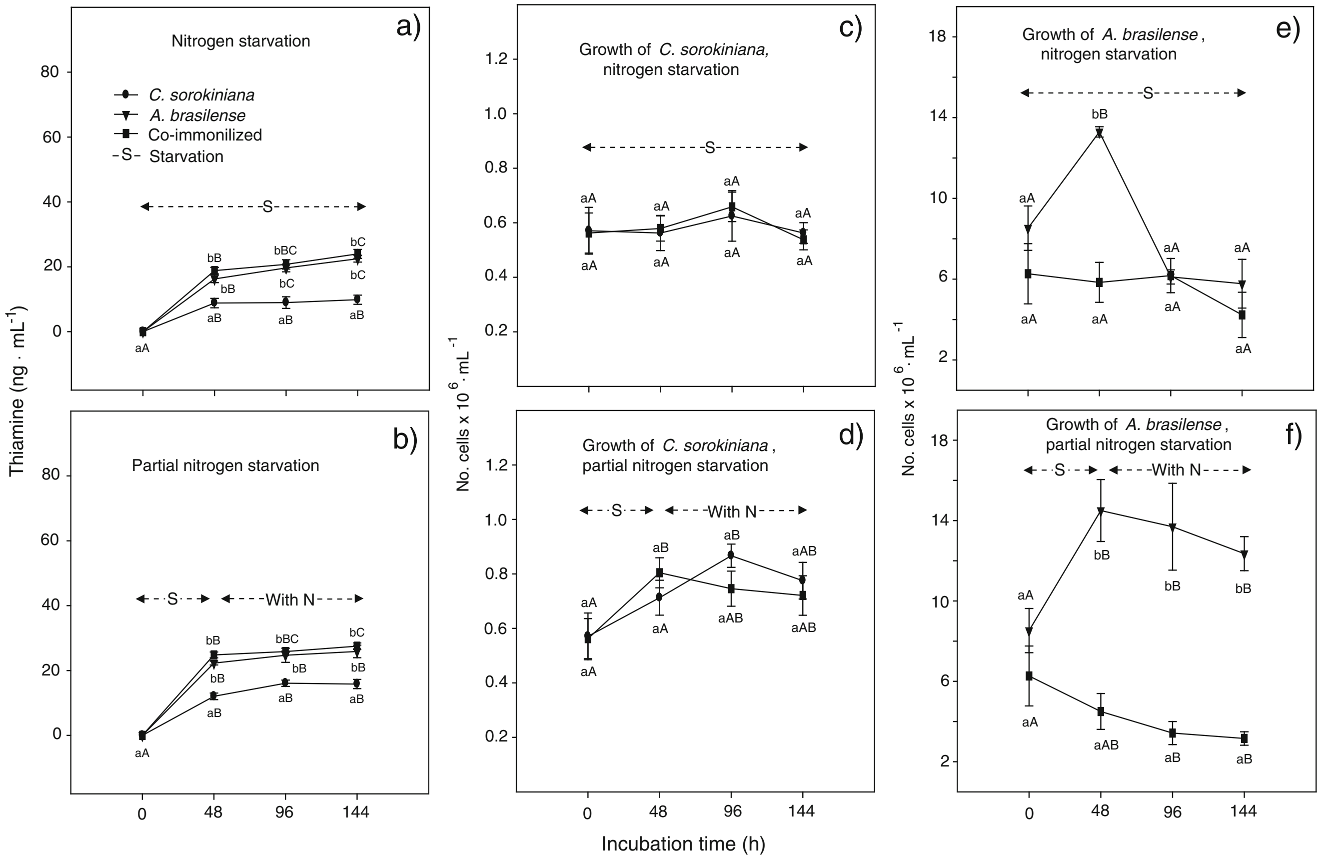

Fig. 3 Comparison of thiamine content in exudates released by *Chlorella sorokiniana* and *Azospirillum brasilense* immobilized separately or immobilized together in alginate beads under nitrogen starvation for the full experiment or partial nitrogen starvation (**a**, **b**) and growth of *C. sorokiniana* (**c**, **d**) and *A. brasilense* (**e**, **f**) under these conditions. Values along curves denoted by different *capital letters* differ significantly using one-way ANOVA and LSD post hoc analysis at $P < 0.05$. Points at each time interval denoted by different *lower case letters* differ significantly at $P < 0.05$ by the same statistical analyses. *Bars* represent SE

was co-immobilized with *A. brasilense*, growth recovered after 144 h, indicating alleviation of stress by *A. brasilense*. Under intense irradiation, the photosynthetic apparatus does not use light energy properly; the excess energy leads to the formation of highly active oxygen molecules (Bar et al. 1995). The interaction between *C. sorokiniana* and *A. brasilense* enhances production of photosynthetic pigments in *C. sorokiniana*, which may improve photosynthesis (de-Bashan et al. 2002). An increase in auxiliary photoprotective photosynthetic pigments in wheat seedlings by *A. brasilense* was reported in wheat plants (Bashan et al. 2006), but not in microalgae. The decrease of concentration of thiamine in exudates may be a consequence of two metabolic processes. Since vitamin excretion is related to cell multiplication (Nishijima et al. 1979), at very high light intensity (for *C. sorokiniana*), the light inhibits growth of *C. sorokiniana* cells as it inhibits production and release of thiamine. Also, thiamine is sensitive to strong light and is degraded (Dănet and Calatayud 1994; Hagen et al. 1991).

Under nitrogen starvation, release of thiamine and growth of *C. sorokiniana* cells were negatively affected. This happened because nitrogen starvation mainly affects photosynthetic efficiency, causing inactivation of the photosystem II in unicellular microalgae (Berges et al. 1996). In our study, the negative effect of nitrogen starvation in the first 24 h was enough to completely stop the growth of *C. sorokiniana*. Even when nitrogen was added 24 h later, the microalgae did not recover. Floreto et al. (1996) reported that the green macroalgae *Olva pertusa*, when starved of nitrogen, was negatively affected, mainly reducing biomass and growth rate. This major stress event probably inhibited the release of thiamine.

Under nitrogen starvation, we did not find thiamine when *C. sorokiniana* was immobilized alone. This occurs because the thiazole ring of thiamine is produced from the amino acid glycine, from which one atom of carbon and one atom of nitrogen are taken (Nosaka 2006). Under nitrogen starvation in the diatom *Thalassiosira pseudonana*, N-containing osmolytes such as proline, homarine, and glycine betaine are replaced by accumulation of dimethylsulfonium propionate, which does not contain nitrogen (Bucciarelli and Sunda 2003). Therefore, we assume that the low release of thiamine during nitrogen starvation in our study results from the absence of precursors of thiamine, such as glycine. Although

Bertrand and Allen (2012) propose that thiamine may enhance the ability of effectively responding to and recovering from nitrogen starvation in phytoplankton, this did not happen in our study. Even when thiamine was present in the exudates of microorganisms in the co-immobilized treatment, growth of *C. sorokiniana* did not recover. Taken together, this shows that nitrogen availability is a primary factor controlling cellular responses in *C. sorokiniana*, surpassing thiamine production and release.

In our study, *A. brasilense* released thiamine under all growth conditions that we tested. The production of B vitamins by this bacterium depends on a carbon source (Rodelas et al. 1993) and pH (Dahm et al. 1993). In our study, *A. brasilense* had the ability to release thiamine in a mineral medium without any external carbon source; however, the release of thiamine was enhanced under acidic pH, but only during the first 48 h. This can be explained since the bacterium can use stored energy compounds such as poly-β-hydroxybutyrate to regulate their metabolism, and this favors establishment and survival in competitive environments under suboptimal conditions (Kadouri et al. 2003). We propose that, because thiamine is an important co-factor in diverse metabolic pathways, the stored carbon ensures production of thiamine when a carbon source is limited.

The exact function of thiamine in this synthetic mutualism is still an open question, currently under investigation. On one hand, both microorganisms are self-producers of vitamin B_1 and can sustain life without external thiamine. Yet, the interaction released thiamine. There are at least two plausible explanations that thiamine has a yet-to-be-discovered function in other metabolic activities that promote microalgal growth and metabolism: (1) enhancing production of indole-3-acetic acid (IAA; Meza et al. 2015a, b), in which thiamine is a known co-factor for IAA production. (2) Thiamine may participate in enhanced carbohydrate metabolism in microalgae. Thiamine is an important co-factor in carbohydrate metabolism in all organisms. Recently, Choix et al. (2012a, b, 2014) show that during this synthetic mutualism, total content of carbohydrates and especially starch and its specific metabolism are significantly enhanced. So far, it is impossible to separate the metabolic effect of each of the partners in this synthetic mutualism experiment. Therefore, our experiments can only show that applying pH stress conditions to the microalgae enhanced release of thiamine. However, we cannot show the direct effect of stressors on thiamine biosynthesis of each microorganism. An effort is currently underway to develop a method to separate the effect of each partner (J.P. Hernandez, personal communication).

One of the main findings of this study is that during the interaction of the two microorganisms, thiamine is always available for both microorganisms, as evident by the release of this vitamin in the exudates. Also, this indirectly indicates a constant production of thiamine. When alleviation of stress in *C. sorokiniana* by *A. brasilense* occurs, this indicates that release of thiamine has recovered. This suggests a yet-to-explore link between stress mitigation in *C. sorokiniana* by *A. brasilense* and thiamine.

Conclusions

These results show that

1. Thiamine is released during synthetic mutualism between *C. sorokiniana* and *A. brasilense.*
2. Larger amounts of thiamine are released specifically during alleviation of a pH stress in the microalgae.

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