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Day and blue light modify growth, cell physiology and indole-3-acetic acid production of *Azospirillum brasilense* Az39 under planktonic growth conditions

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Running head: Light effects on *A. brasilense* Az39

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Abstract

Aim.

In this work we evaluated the effects of light on growth, cell physiology and stress response of *A. brasilense* Az39, a non-photosynthetic rhizobacteria, under planktonic growth conditions.

Methods and results.

Exponential cultures of Az39 were exposed to blue (BL), red (RL) and daylight (DL) or maintained in darkness for 24, 48 and 72 h. The biomass production and indole 3-acetic acid (IAA) biosynthesis increased by exposition to DL. Conversely, BL decreased IAA concentration through a direct effect on the molecule. The DL increased superoxide dismutase activity, hydrogen peroxide and thiobarbituric acid reactive substances levels, but the last one was also increased by BL. Both DL and BL increased cell aggregation but only BL increased biofilm formation.

Conclusions

We demonstrated that both BL and DL are stress effectors for *A. brasilense* Az39 under planktonic growth conditions. The DL increased biomass production, IAA biosynthesis and bacterial response to stress; while, BL induced cell aggregation and biofilms formation, but decreased the IAA concentration by photooxidation.

Significance an impact of the study

BL and DL changes growth capacity, cell physiology and plant growth promotion ability of *A. brasilense* Az39 and these changes could be considered to improve the production and functionality of biofertilizers.

Keywords: *Azospirillum*; indole-3-acetic acid; cell aggregation; biofilms; oxidative stress; light environment

Introduction

There are many light-induced photosynthetic bacteria that rely on light acquisition to fulfill their life cycle. However, light can be either beneficial or harmful for non-photosynthetic microorganisms (Van Der Horst and Hellingwerf, 2004). Daylight contains a wide range of energy with wavelengths ranging from radio waves (30 km to 300 mm) to rays X (90 nm to 10 pm). Despite this, the energies derived from the visible and infrared regions of the spectrum maintain life in most biological systems (Kraiselburd *et al.* 2017; Schumacher, 2017).

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The lethal or detrimental effects for life are concentrated in the regions of violet (380-450 nm) and blue (450-495 nm) while the beneficial effects are concentrated in the region of red (660-730 nm). The role of light-induced proteins in photosynthetic bacteria is well known (Giraud *et al.* 2002); however, the functions of such proteins are still a matter of study in non-photosynthetic bacteria (Altschul *et al.* 1997).

Members of genus *Azospirillum* are gram-negative, alpha-proteobacteria with the ability to colonize over a hundred plant species (Bashan and de-Bashan, 2010). Despite being defined as a non-photosynthetic bacterium, sequences coding for phytochromes (PHY) and bacteriophytochrome heme-oxygenase proteins have been identified in the *A. brasilense* Az39 genome (Rivera *et al.* 2014). These proteins would be responsible for the bacterial capacity to perceive and respond to daylight or/and particularly to the red light (630 nm). Although the effects of red light on growth, physiology and life style on photosynthetic bacteria are well known, there is very little information about the effects of such wavelength in non-photosynthetic ones. In *Deinococcus radiodurans*, an extremophilic and radiation resistant bacterium, phytochromes are involved in photooxidative stress avoidance by inducing carotenoid biosynthesis (Davis *et al.* 1999). However, in *A. brasilense* Sp7 these proteins have been associated to red light tolerance by carotenoids independent pathways (Kumar *et al.* 2012). This publication represents the unique reference showing the effect of light on *A. brasilense*, with a particular emphasis on red light. On the other hand, the genome sequence analysis of *A. brasilense* Az39 has not revealed the presence of any coding sequences for other photoreceptors, such as yellow photoactive proteins (PYP or xantopsins), Light Oxygen or Voltage (LOV) proteins, cryptochromes or Blue-Light Sensing Using Flavin (BLUF) proteins, so we have no molecular or physiological references to explain the behavior of this bacterium in the presence of other light ranges. A previous study performed in our laboratory has shown that if *A. brasilense* Az39 is cultured under solid or semisolid culture medium conditions, the exposure to BL or DL was lethal and inhibited bacterial growth and swimming motility (Molina *et al.* 2020). By contrast, the exposure to RL did not show any harmful effects and bacteria showed a similar behavior than those cultured under D (Molina *et al.* 2020).

Members of the genus *Azospirillum* are considered as metabolically versatile because they have been isolated from a wide range of environmental conditions (Reis *et al.* 2015). This versatility would reside in their genotypic and phenotypic plasticity and their ability to respond to stressing environmental conditions, such as high and low temperatures, pH variations, low nutrient availability, metal concentrations, pesticide residues and herbicides among other environmental factors (Bashan *et al.* 2004). The exposition to environmental stress induces an increase in the concentration of peroxide, superoxide, hydroxyl radical and singlet oxygen, defined as reactive oxygen species (ROS). ROS are natural products of normal oxygen metabolism and are important molecules for bacteria signaling and homeostasis (Imlay, 2013). However, their increase resulting from stress

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conditions can become deleterious by reacting with DNA or RNA, lipids (lipid peroxidation), and proteins, provoking oxidative damage (Imlay, 2003). Enzymes such as catalases (CAT) and superoxide dismutases (SOD) ameliorate the damaging effects of hydrogen peroxide and superoxide, respectively (Gratão *et al.* 2015). *A. brasilense* has been one of the most widely used plant growth promoting rhizobacteria in agriculture in the last forty years worldwide (Cassán *et al.* 2020). The selection of *A. brasilense* strains for biofertilizers production mostly depends on the bacteria ability to grow, fix atmospheric nitrogen and produce phytohormones, mainly indole-3-acetic acid (IAA) (Tien *et al.* 1979). Additionally, the cellular behavior and the bacterial capacity to form aggregates, flocs or other resistance cellular structures (i.e. cystic forms) that would favor their survival in unfavorable conditions as well as their capacity to respond to environmental stresses, are important factors taken into account for selection (Sadasivan and Neyra, 1985). Several physical (temperature or gas concentration), physiological (nutrient availability) or biochemical effectors (presence of signal molecules) in the medium, regulate bacterial growth, auxins biosynthesis (Molina *et al.* 2018), as well as the cell behavior through cell aggregates and biofilms formation (Karatan and Watnick, 2009), but no references are available in relation to the effects of light on these bacteria.

Our hypothesis claims that light acts as a strong environmental stress effector and changes bacterial physiology and some of the most significant attributes related to the *Azospirillum*´s biofertilizers strain selection. Therefore, the main objective of this paper was to evaluate the effects of DL, RL and BL on bacterial growth, cell physiology and plant growth promotion capacity of *A. brasilense* Az39 under planktonic growth conditions.

Material and methods

Biological material and growth conditions

A. brasilense Az39 was obtained from the Instituto de Microbiología y Zoología Agrícola (IMyZA) at the Instituto Nacional de Tecnología Agropecuaria (INTA) (Castelar, Buenos Aires, Argentina). The phenotypic variant Az39 pFAJ64, obtained by Rivera *et al.* (2018) according to the methodology proposed by Vanstockem *et al.* (1987) was also used. This strain contains the plasmid pFAJ64 with a tetracycline resistance (Tc) cassette and an *ipdC*-*gus*A fusion and it was constructed to quantify the expression of *ipdC* which codes for the enzyme Indole-3-pyruvate decarboxylase (EC:4.1.1.74) by the β-glucuronidase reaction (Ona *et al.* 2005). Both *A. brasilense* Az39 and the transformed strain (pFAJ64) were grown in sterile Luria Bertani broth medium (Bertani, 1951) in darkness until OD_{595} reached 1.5 at 36°C overnight. Then 40 µl from this pre-inoculum were transferred into an Erlenmeyer flask containing sterile LB medium, which was incubated in a dark growth chamber at 36°C with 200 rev min^{-1} shaking until exponential growth phase corresponding to OD_{595} 0.1 was

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reached. The inoculum was then distributed in equal volume (20 ml) into sterile polyestyrene Petri dishes with UV radiation absorption at 280-290 nm (Deltalab, Spain). This experimental model was performed to increase the exposition of culture medium to the different light conditions. Then, inoculated plates were independently exposed to experimental conditions related to the presence or absence of light: daylight (DL) [56 $\mu W/mm^2$], generated by a 75 watt incandescent light lamp (General Electric, USA), blue light PAR 38 (BL) [11 $\mu W/mm^2$] and red light PAR 38 (RL) [13.9 $\mu W/mm^2$], generated by 450 nm and 660 nm LED lamps respectively (OSRAM, Germany), and cultured at 36°C for 24, 48 and 72 h in static conditions. In all cases, a control treatment was performed by maintaining the same growth media and growth conditions in darkness with double aluminum foil. A Vernier, Spectro Vis-Plus spectrophotometer (USA) was used to confirm the wavelength values for each light source, and their intensity was measured using a Tenmars TM-201 lux meter (Taiwan).

Biomass production and cell viability

Biomass production was measured at different incubation times by optical density at 595 nm (Zeltec ZL5000P, Argentina). The number of viable cells was obtained in agar plates containing LB culture medium, modified by the addition of 15 ml l^{-1} Congo Red indicator (LB-CR) (Molina *et al.* 2014), through the microdroplet technique (Rivera *et al.* 2014). The colony forming units per milliliter (CFU ml^{-1}) were calculated considering dilution and inoculation factors [CFU ml^{-1}= dF x iF x 50 (dF: dilution factor; iF: inoculation factor)] and converted to log10 for the figures. Inoculated plates were incubated at 36°C for 72 h and the procedure was performed in triplicate.

Indole-3-acetic acid biosynthesis

IAA production

Both, *A. brasilense* Az39 and Az39 pFAJ64 cultures obtained as previously described were centrifuged at 13.500 *g* for 10 min to collect the supernatant. Identification and quantification of IAA was conducted by reverse-phase HPLC (Rivera *et al.* 2018). Briefly, an Aligent 1200 Series HPLC system with Quaternary Pump, which features an Aligent Eclipse XDB-C18 column (4.6 mm diameter, 150.0 mm length and 5.0 µm particle size), was used at a flow rate of 1 ml min^{-1}. Elution was performed with a mixture of H_2O and MeOH (60:40) containing 0.5% acetic acid, and monitored at 280 nm. A non-inoculated culture medium was used as control. The IAA production under similar experimental conditions was confirmed in *A. brasilense* Az39 wild type strain. The concentration was expressed as IAA µg ml^{-1}.

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ipdC gene expression

The indole-3-pyruvate pathway (IPyA) has been proposed as the main route for IAA biosynthesis in *A. brasilense*, with *ipdC* being the key regulator gene (Costacurta *et al.* 1994). The expression of *ipdC* was analyzed in *A. brasilense* Az39 pFAJ64 by the β-glucuronidase activity in microtitre plates, using GusA extraction buffer and p-nitropheny L-b-D-glucuronide as substrate (Jefferson, 1987). Cultures obtained as previously described were centrifuged at 13.500 *g* for 10 min to collect the supernatant. Activity was expressed in Miller units (Miller, 1972) and represented the mean of three measurements and three biological replicates.

Indole-3-acetic acid degradation

The direct effect of light on the IAA molecule was evaluated. Petri dish plates containing 20 ml of no-inoculated LB medium and modified by the addition of an IAA solution to obtain a final concentration of 10 µg ml^{-1} were used. The plates containing the cultured medium modified with the hormone were exposed to white, blue, and dark light for 24, 48 and 72 h at 36°C as described above. After exposition, the IAA concentration was determined by HPLC as previously described.

Cellular behavior

Cell aggregation

Cell aggregation was determined according to Madi and Henis (1989), applying modifications according to Burdman *et al.* (1998). The volume contained in each plate exposed to different light sources or darkness conditions was transferred into a conical tube and allowed to stand for 20 min. Then, turbidity was measured at 540 nm using a Zeltec ZL5000P spectrophotometer (OD1). The culture was homogenized for 1 min and the turbidity was measured again (OD2). The aggregation percentage was calculated according to the following equation %AP $(OD_2 - OD_1) \times 100/OD_2$

Biofilm formation

The biofilm forming capacity was quantitatively analyzed by measuring the number of cells attached to a glass disk incorporated within the Petri dish containing the bacterial culture (Prouty *et al.* 2002), using the crystal violet staining method proposed by O'Toole and Kolter (O'Toole and Kolter, 1998) with modifications. At each exposure time, the glass was taken under aseptic conditions, washed with 1 ml of NaCl (0.9% w/v) and treated with 1 ml of crystal violet indicator (0.1% w/v) over 20 min. Then, the glasses were washed 3 times with NaCl (0.9% w/v). Biofilm formation was quantified by adding 1 ml of 95% ethanol to each crystal violet stained glass. The OD_{560} was measured in a Zeltec ZL5000P spectrophotometer (Zeltec, Argentina).

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Photooxidative stress and antioxidant response

Hydrogen peroxide

The hydrogen peroxide content was estimated spectrophotometrically by its reaction with potassium iodide (KI) (Alexieva *et al.* 2001). *A. brasilense* Az39 was grown in LB medium as previously described. Cultures were centrifuged at 12.000 *g* for 10 min and washed twice with a physiological solution. The pellets were resuspended in 50 mmol l^{-1} potassium phosphate buffer (pH 7) and 10% (v/v) trichloroacetic acid (TCA) and sonicated (amplitude: 80; time: 2 min; pulse: every 6 s). The extract was centrifuged for 15 min at 4ºC and 2.000 *g*. The supernatant (0.16 ml) was mixed with 0.16 ml of 100 mmol l^{-1} K-phosphate buffer and 0.68 ml reagent (1mol l^{-1} KI w/v in water). The reaction was kept in the dark for 1 h after which the absorbance at 390 nm was recorded. The hydrogen peroxide content was estimated from a standard curve prepared with aliquots of 1 mmol l^{-1} H_2O_2.

Lipid peroxidation

Lipid peroxidation evaluation was performed according to Heath and Packer (1968) with some modifications. In this reaction, thiobarbituric acid (TBA) reacts with the aldehyde group of malondialdehyde (MDA) (final product of lipoperoxidation) and other aldehyde reactive substances (TBARs) to produce a pink compound with maximum absorbance at 532 nm. *A. brasilense* Az39 was grown in LB medium as previously described. Cultures were centrifuged at 12.000 *g* for 10 min and washed twice with physiological solution. The pellets were resuspended in 50 mmol l^{-1} potassium phosphate buffer (pH 7) and 10% (v/v) trichloroacetic acid (TCA) and sonicated (amplitude: 80; time: 2 min; pulse: every 6 s). The extract was centrifuged for 15 min at 4ºC and 2.000 *g*. The TBARs content was determined from the supernatant by the use of a reaction mixture containing 100 μl of supernatant, 100 μl of 0.1 mol l^{-1} EDTA and 600 μl of 1% (w/v) thiobarbituric acid (TBA) in 50 mmol l^{-1} NaOH. The samples were heated at 95°C for 15 min, then the absorbance was measured at 532 nm, corrected by the nonspecific absorption at 600 nm. The concentration was calculated using an extinction coefficient of 155 mmol l^{-1} cm^{-1}.

Antioxidant enzyme

Bacterial cultures were centrifuged as described earlier. The pellets were resuspended in 2 ml of extraction buffer (50 mmol l^{-1} phosphate buffer, 1 mmol l^{-1} Na-EDTA pH 7.5), sonicated (amplitude: 80; time: 2 min; pulse: every 6 s) and centrifuged again. Supernatants were used to determine enzyme activities. SOD activity (EC 1.15.1.1) was determined through the technique proposed by Beauchamp and Fridovich (1973), using

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nitrotetrazolium blue (NBT) in the presence of riboflavin. A volume of 1 ml reaction mixture containing 0.54 μmol l^{-1} EDTA, 75 μmol l^{-1} NBT, 777 μmol l^{-1} methionine and 50 mmol l^{-1} potassium phosphate buffer (pH 7.8), 4 μmol l^{-1} riboflavin and 5 μg of the protein extract was placed under fluorescent light for 15 min. The specific SOD activity was determined by spectrophotometry at 560 nm and was expressed as U mg^{-1} protein. One unit of SOD activity was defined as the amount of enzyme required to inhibit NBT reduction by 50%. CAT activity (EC 1.11.1.6) was determined using the technique proposed by Aebi (1984) in a reaction mixture containing 0.1 mg of enzyme protein, 50 mmol l^{-1} potassium phosphate buffer (pH 7.4) and 12.5 mmol l^{-1} H_2O_2. The specific activity of CAT was determined by the decomposition of H_2O_2 by measuring the decrease in absorbance at 240 nm. To estimate the enzyme units, the molar extinction coefficient of H_2O_2 (43.6 mol l^{-1} cm^{-1}) was used. Total protein content was evaluated according to Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

All assays were performed in triplicate with three repetitions for each growth condition. Data obtained were tested with ANOVA and Honestly-Significant-Difference (HSD) Tukey, with a 95% confidence level. Infostat software (Universidad Nacional de Córdoba, Argentina) was used to carry out the analyses, and the graphs were designed using GraphPad Prism 5.0 software (GraphPad Software, USA).

Results

Biomass production and cell viability

The exposure of *A. brasilense* Az39 cultures to daylight (DL) caused a significant increase (29%) in biomass production in comparison with the control treatment (darkness, D) after 24 h exposure (Figure 1a). At 48 h, there were no significant differences, but an increase trend of 6% was observed in the case of DL exposure. This trend remained significant and it was about 11% after 72 h exposure. Figure 1b shows that bacterial exposure to blue light (BL) caused a significant increase of 12% in biomass production in comparison with D, but it was reversed after 48 h where D showed a non-significant increase of 5%. There were no significant differences between BL and D after a 72 h exposure. Figure 1c shows that bacterial exposure to red light (RL) only produced a significant difference of 10% biomass after 48 h of exposure in comparison with D. The cell viability (CFU ml^{-1}) was similar under all experimental conditions and there were no statistically significant differences between the treatments (Figure S1). In summary, only the *A. brasilense* Az39 exposure to DL caused an increase in the biomass production in comparison with the control (D) or the other treatments (BL

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and RL), along the assay, but this behavior was not correlated with a significant increase in the number of cells in the culture medium.

Indole-3-acetic acid biosynthesis

Some environmental factors affect IAA biosynthesis in *A. brasilense*. In this work, the effects of light on the production and accumulation of IAA in the culture medium were evaluated, as well as the incidence of this factors on the expression of *ipdC*, the key gene of the hormone biosynthesis pathway. As seen in Table 1, the IAA concentration in *A. brasilense* Az39 pFAJ64 cultures exposed to DL was 1.8 times higher than the concentration reached in the control maintained in darkness (D) after 24 h exposure. The same treatment caused 2 times increase in the IAA concentration in comparison to the culture exposed to RL which, in turn, presented a negative difference (14%) in relation to D. In the case of BL caused a 1.4 times higher bacterial capacity to produce the hormone, in comparison with D. After 48 h, DL treatment showed a maximum increase of 2.1 times in IAA concentration in comparison with D. In the case of RL, the IAA production was 33% lower than that in the case of D. For BL, there was no significant difference in comparison to the control kept in dark conditions. After 72 h of exposure to DL, the IAA concentration maintained a constant increase (2.1 times higher) in comparison to D. In the case of RL there were no significant differences in comparison with the D treatment. On the other hand, cultures exposed to BL showed a 2.5 and 2.2 times lower in IAA concentration in comparison to the same treatment at 48 h and the control treatment (D) at 72 h. Taking into account this result, the stability of IAA molecule in non-inoculated culture medium exposed to light conditions was evaluated and it is summarized in Figure S2. The non-inoculated culture medium supplemented with IAA 10 μg ml^{-1} and exposed to DL and BL showed a lower IAA concentration than in D, in which the hormone concentration was higher and unalterable throughout the experiment. The BL reduced the IAA concentration by 30 and 70% after 48 and 72 h exposure, in comparison to the control D.

As seen in Table 1, the bacteria exposure to DL increased by 1.7 times the *ipd*C gene expression compared to D. The expression was maximum after 48 h, but it remained constant until 72 h, when it was 2.1 times higher than D. When the bacteria were exposed to BL, the *ipdC* expression was 1.4 times higher in comparison to D after 24 h; however, later on, no significant differences were observed regarding this treatment. Finally, when bacteria were exposed to RL, the gene expression was increasing with time, but always remained below D. Summarizing, DL was the only treatment causing a significant increase in both the *ipdC* expression and the IAA concentration in the culture medium. In the case of BL, RL and D, the *ipdC* expression was similar, but a significant reduction in the IAA concentration was found in BL (Figure S2). This behavior was also slightly observed when the bacteria were exposed to DL. In summary, DL increased both the expression of *ipd*C and

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the accumulation of IAA in the culture medium, while BL, although it increased the expression of *ipd*C, reduced the concentration of the hormone in the culture medium through a direct effect on the molecule.

Cellular behavior

In certain bacterial genera, cell aggregation and biofilm production are related to the bacteria response to environmental stress. In this work, the effects of light on both cell aggregatt and biofilm production were evaluated in *A. brasilense* Az39. In Figure 2, the DL and BL treatments resulted in a significant increase in the cellular aggregation percentage of *A. brasilense* Az39 in comparison to D. This increase was proportional to the exposure time, since after 24 h there was an increase of 40 and 66% for DL and BL respectively. After 72 h of exposure, these treatments caused a maximum difference of 59% and 78% in comparison with D. In the case of RL, the lowest percentage of aggregation was observed in comparison with DL and BL, and the behavior of the bacteria exposed to this wavelength was generally similar to D.

Figure 3 shows that the production of biofilms was time-dependent in all cases, reaching the highest production after 72 h of culture, regardless of the light treatment. Under dark conditions, the formation of biofilms was 80% higher than in the other treatments after 24 h; however, this difference disappeared after 48 h of incubation. Cultured exposed to BL showed a biofilm production lower than the D after 24 and 48 h exposure. However, BL exposure caused a maximum biofilm production after 72 h with a 23% increase in comparison to D. The DL and RL induced the lowest biofilm production compared to the rest of the treatments. In these last two conditions, the production of biofilm was on average about 30% lower than in the control at 48 and 72 h. In summary, the exposure to BL and DL caused an increase in the production of cell aggregates in planktonic cultures of *A. brasilense* Az39, but in the case of BL also caused an increase of the biofilm production.

Photooxidative stress and antioxidant response

As we have previously mentioned, BL and DL are lethal and inhibit the *A. brasilense* Az39 growth in solid culture medium. On the contrary, under planktonic growth conditions, bacteria are able to grow and develop. Analyzing the oxidative state and the antioxidant response of Az39 to such conditions allow us to understand the bacterial perception and cell response to light stress. Figure 4a shows the production of hydrogen peroxide in cultures of *A. brasilense* Az39 exposed to different wavelengths compared to the treatment D. The DL caused the highest production of hydrogen peroxide, being 6 times higher than the values reached in D. A similar behavior was observed in the case of BL, where the peroxide production was 1.8 times higher than in

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D. Finally, in the case of RL, the production of hydrogen peroxide was about 0.6 times lower than that produced in D and this difference was statistically significant. In summary, both BL and DL caused an increase in the production of hydrogen peroxide, unlike RL in which the levels were lower than in treatment D. Figure 4b shows that the production of malondialdehyde (TBARs) in the DL treatment was 1.8 times higher than in BL. No detectable levels of this compound was recorded in the RL or D conditions. On the other hand, the RL did not produce significant changes and presented a behavior similar to that observed in D.

As shown in Figure 5a, the catalase activity (CAT) of cultures exposed to DL, BL and RL treatments was lower than that of cultures kept in D. The CAT activity of cultures exposed to D was 35% higher than the CAT activity of cultures exposed to DL and RL; while in the case of BL, the CAT activity was 52% lower than the one measured in the control treatment. The superoxide dismutase (SOD) activity is summarized in Figure 5b and presented an opposite pattern to the one observed for the CAT activity, where the cultures exposed to different types of light presented a higher activity than in D. When the bacteria were exposed to DL, the enzyme activity was 28% higher than when exposed to D and this difference was statistically significant. In the case of BL and RL, SOD activities were respectively 33% and 29% higher than in the D condition, although those differences were not statistically significant. In summary, BL and DL produced an increase in the hydrogen peroxide and malondialdehyde concentrations indicating an oxidative stress condition. On the other hand, at the level of antioxidant response, increases in SOD but not CAT activity were induced by DL (significant) and BL (not significant).

Discussion

A.brasilense, is one of the most important rhizobacteria used in agriculture worldwide, but the information about the bacterial response to light is limited or almost null. In the previous paper by Molina et al. (2020) we studied the effects of light on the growth and viability of *A. brasilense* Az39 in solid culture medium and we verified that both DL and BL were lethal for the bacteria. In solid culture medium, light directly affects both components of the culture medium and bacteria cells deposited at the surface producing a bigger exposure of them to the light. Bacterial death due to the chemical modification of the culture medium has been previously explained by Boyd et al. (2019). In this article, we found that BL and DL were not lethal for *A. brasilense* Az39 and cells were able to grow, but showing clear indications of stress. Under liquid culture medium (planktonic) conditions, bacteria are immersed and surrounded by water and nutrients such as amino acids, vitamins and sugars. This matrix decreases the exposure of the bacteria to light by adsorption (i.e. nutrients) and refraction (i.e. water) processes. Similar observations were previously reported by Elmnasser et al. (2007) for other bacteria such as *Listeria monocytogenes, Pseudomonas fluorescens* and *Photobacterium*

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phosphoreum. When these bacteria were exposed to light in solid culture medium, there was a drastic decrease in cell viability; however, under planktonic growth conditions, they showed lower sensitivity to the light.

In this work we evaluated the photooxidative stress and the antioxidant response of *A. brasilense* Az39. The bacteria exposure to DL increased the levels of H_2O_2 in comparison to the control treatment and this parameter was also associated with an increase in TBARs levels, not only when exposed to DL, but also to BL. From these results, it could be proposed that DL, and to a lesser extent, BL, would be effectors of oxide-reduction (redox) changes in the metabolism of the bacterium and consequently increase the generation of reactive oxygen species (ROS). Considering both the H_2O_2 and malondialdheyde production, the DL and to a lesser extent BL modify the oxidative state of *A. brasilense* Az39 membranes, in what seems to be a direct consequence of a lipid peroxidation process. Membrane lipids are the first molecules to be affected by an oxidative burst because free radicals directly attack polyunsaturated fatty acids in the membranes and thereby initiate lipid peroxidation. TBARs are dialdehydes formed as secondary metabolites during the oxidation of polyunsaturated fatty acids and are used as indicators of lipid peroxidation and oxidative stress (Dourado and Cesar, 2015; Esposito *et al.* 2015). SOD and CAT are enzymes involved in the protection of cells against excessive production of O_2- and H_2O_2 respectively (Cabiscol *et al.* 2000). In a general way, SOD is critical for antioxidant responses to herbicides, heavy metals, and other biotic and abiotic factors; however, little is known about their activity in bacteria in the presence of light. In relation to the SOD activity, an increase was detected when exposed to DL; while in D the levels of this enzyme were lower. The H_2O_2 can be produced from SOD activity during aerobic cell growth or as a product of redox reactions with flavoproteins. In relation to CAT activity, it was superior in D compared to treatments exposed to light, including DL. From this observation it could be assumed that the H_2O_2 detoxification system is not active through the catalase activity. Both catalases and peroxidases are the primary degraders of H_2O_2 in many bacteria. However, it has been proposed that a wide variety of additional enzymes could fulfill similar functions such as thiol peroxidase, comigratory bacterioferritin, glutathione peroxidase, cytochrome c peroxidase, and rubreritrins (Mishra and Imlay, 2012). Each of these enzymes is able to degrade H_2O_2 *in vitro*, but their contribution *in vivo* remains unclear. According to Méndez-Gómez et al. (2016), CAT and SOD activity are not always expressed simultaneously in *Azospirillum*. They reported that SOD activity of *A. brasilense* was modified by exposure to plant cell wall effectors, but CAT activity remained unchanged. Bacteria cells generally have multiple detoxification systems sometimes working alternately or decoupled, so that the absence of one is hidden by the presence of another; a single enzyme would not be enough (Mishra and Imlay, 2012). Overall, the redox status indicators analysis showed the detrimental effects of DL and BL on bacterial metabolism, and allowed to explain further responses induced by the exposition to these distinctive light sources.

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Bacteria respond to adverse environmental factors such as UV radiation, heavy metal toxicity, pH changes, dehydration, salinity, and the presence of antibiotics or antimicrobial agents through the formation of specialized structures called biofilms (Barragan et al. 2011; Gilbert et al. 2002). Significant differences in the biofilms formation were observed between the treatments under our experimental conditions. The longest time (72 h) and BL exposure showed an increase in the biofilms formation, which was directly correlated with the cell aggregation phenomenon. For most of the studied cases, the ability to form biofilms is related to an increased virulence (colonization capacity) or bacterial competition; while in the case of *Azospirillum* the biofilms formation would be also related with the bacterial capacity to overcome environmental stress conditions, such as light. The *A. brasilense* capacity to increase biofilm production by exposure to BL seems to be a particular characteristic of these bacteria. Kahl et al. (2020) reported a decrease in biofilm production in *P. aeruginosa* PA14 due to prolonged exposure to BL. Chebath-Taub et al. (2012), reported that BL produced a delayed antibacterial effect on *Streptococcus mutans*, although the bacterium did not modify its ability to produce biofilms.

Under environmental stress conditions, *Azospirillum* induces aggregation and flocculation in liquid culture media (Pereg, 2015). The results presented in this work demonstrate an increase in the percentage of cell aggregation after exposure to BL and DL. The cell aggregation process is closely related to the nutritional condition in which the microorganisms are found, but it also depends on certain environmental signals to which it is exposed (Burdman *et al.* 2000). In the case of *A. brasilense*, there is concrete evidence on the role of extracellular polysaccharides (exopolysaccharides, EPS and capsular polysaccharides, CPS) in the process of cell aggregation (Burdman *et al.* 2000). The results presented in this work demonstrate an increase in the percentage of cell aggregation after exposure to BL and DL. Previous studies have shown that cell aggregation phenomenon in *A. brasilense* is mediated by the composition of monosaccharides present in EPS (Burdman *et al.* 2000) and by proteins, such as the type of lectins (Nikitina *et al.* 2001). Despite the concentration of total EPS was not significantly modified by the exposure to DL and BL (data not shown); but neither EPS composition or lectins concentration were measured in our experiments to confirm this possibility. Taking into account that cell aggregation is also related to the presence of both type IV or Tad pili in certain microorganisms (Shelud'ko and Katsy, 2001) and considering that *A. brasilense* have Tad pili (Wisniewski-Dyé *et al.* 2011) we consider this structure would be partly responsible for the cell aggregation under DL o BL exposition. Interestingly *Sulfolobus solfataricus* showed an increase of cell aggregation as a result of the pili formation by exposure to light (Fröls *et al.* 2008).

The environmental stress induces significant morphological changes at the cellular level in bacteria, including loss of mobility, increased accumulation of reserve substances, and production of exopolysaccharides,

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suggesting that cell surface remodeling is a strategic response to cope with a stressful condition (Bible *et al.* 2015). The bacterial biomass production was increased by the exposition to DL, but it did not correlate with an increase in the number of viable cells (CFU ml^{-1}) in the culture medium. This behavior was opposite to that previously reported by Molina *et al.* (2020) using the same culture medium, but in solid state, where DL and BL where lethal for *A. brasilense* Az39. To understand this difference, we must consider that absorbance not only depends of the bacterial cells, but also depends on the presence of other elements in the culture medium, such as exopolysaccharides (EPS), lipopolysaccharides (LPS), proteins, aggregates and other compounds released by bacteria into the culture medium as a result of their growth. Particularly, *Azopirillum* produces and releases into the surrounding medium carbohydrates, polysaccharides (exo- and lipo-), and proteins, among other components (Del Gallo and Haegi, 1990). In this work we did not find significant differences in the amount of exopolysaccharides produced by *A. brasilense* Az39 by exposure to BL or DL, so the increase in the optical density (biomass) could depend of additional components or metabolites produced and released by the bacteria into the culture medium. The cell aggregation were increased by exposure to BL and DL and biofilm production in BL so the cellular material responsible for cell adhesion would also been increased.

Indole-3-acetic acid (IAA) is the phytohormone responsible for root growth and development promotion after inoculation with *A. brasilense* (Cassán *et al.* 2014). Recently, we have proven that both the cell viability and the ability of three strains of *A. brasilense* including Az39 to produce and accumulate IAA in the culture medium are modified by the presence of different biotic and abiotic stress effectors, among which are considered the presence of several amino acids (Rivera *et al.* 2018), sodium salts (ionic stress), polyethylene glycol (osmotic stress), or temperature and light, among others (Molina *et al.* 2018). There are other reports showing the effects of several effectors on the biosynthesis of IAA in *A. brasilense* (Lucy *et al.* 2004). In fact, it has been proposed that IAA could act as a positive effector on the regulation of gene expression in this bacterium. *Azospirillum* is able to perceive the hormone in the culture medium and induces the expression of certain genes, including those related with IAA biosynthesis, such as the *ipdC* gene in a *feed-forward* model (Van Puyvelde *et al.* 2011). In other reports, the synthesis of the hormone was positively dependent of the amino acid L-tryptophan, which together with the presence of L-phenylalanine caused an increase in the *ipdC* gene expression (Rivera *et al.* 2018). Additionally, the deficiency of carbon and oxygen in the culture medium, as well as the entrance to the stationary phase by the microorganism are considered as positive effectors for the IAA biosynthesis (Ona *et al.* 2005). Summarizing, any environmental effector that modifies the ability of *A. brasilense* to synthesize IAA could modify its ability to promote plant growth. In this work we reported that both the expression of *ipdC* and the IAA concentration were increased by the exposure to DL in *A. brasilense* Az39. On the contrary, in the presence of BL, although the bacterium increases the *ipd*C expression, a lower

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concentration of the hormone was found in the culture medium, as a result of a direct photo-oxidative effect on the molecule (Yamakawa *et al.* 1979). Considering the inability of the bacterium to degrade the hormone (Rivera *et al.* 2018) and the reduction of the IAA concentration observed after 72 h exposure to both BL and DL, we analyzed the IAA stability in non-inoculated culture media exposed to light. It was observed that when exposed to blue light, the IAA concentration in the medium was inversely proportional to the incubation time, so it could be inferred that the stability of the molecule was directly affected and resulted in the destruction of this compound in the culture medium (Yamakawa *et al.* 1979). Summarizing, the exposure of *A. brasilense* Az39 to any wavelength increased its ability to produce IAA after different exposure times, but only day light significantly increased the production of this hormone with time.

Light is a beneficial environmental effector for photosynthetic microorganisms (Biebl and Wagner-Döbler, 2006). However, in those who are not able to perform this process, the impact of light on their metabolism and lifestyle can range from beneficial to damaging or even lethal. This behavior supposes the capacity of such bacteria to perceive the light stimulus and to define a specific response. Thus, the light signal perception should depend on the presence of specialized molecules. Although a true photosynthetic apparatus is not available in non-photosynthetic bacteria, there are light-receiving structures, generally defined as photoreceptors with an associated role in the metabolism. The genome sequence of *A. brasilense* Az39 contains two genes for the synthesis of photoreceptor proteins fitting in the group of phytochromes (EC: 2.7.13.3), regulated by RL (630 nm) (Phy 1 ABAZ39_30745 and Phy 2 ABAZ39_31245). However, according to previous results (Molina *et al.* 2020) and to those generated in this work, bacterial exposure to RL did not generate any detectable change and showed similar behavior than to the control D. In contrast, both DL and BL were responsible for most of the changes observed, but paradoxically, there are no sequences for BL receptor proteins in the genome of this bacterium. Kumar *et al.* (2012) reported that a mutant of *A. brasilense* Sp7, deficient in the expression of the phytochrome BphP1, had a lower biomass production capacity in rich liquid medium when exposed to RL in comparison with the wild type strain, for which no significant differences were observed by exposure to RL in comparison with the control in D. From these observations, the authors claimed the ability of the phytochrome BphP1 to regulate photodynamic stress in this bacterium. *A. brasilense* Sp7, contains 2 phytochromes sequences annotated as AbBphP-1 and AbBphP-2. One of the sequences (AbBphP-1) is homologous with AbrBphP-1 in the genome of Az39 and AtBphP-1in the genome of *A. tumefaciens* C58 (model strain). According to the *in silico* analysis these sequences encode for canonical phytochromes proteins activated by RL (Lamparter *et al.* 2017). Furthermore, Kumar *et al.* (2012) confirmed this capacity for AbBphP-1 by spectral characterization. Further experiments will be developed to confirm if ABAZ39_31245 or AbBphP-2 of Sp7 are activated by far red light (FRL) and considered a Bathy type phytochromes (Rottwinkel *et al.* 2010).

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This is the first report showing the effects of DL and BL on planktonic cultures of *A. brasilense*, one of the most used plant growth promoting rhizobacteria in agriculture, worldwide. The biofertilizers production and functionality require the bacterial capacity to grow in liquid formulations, survive under stress conditions and promote the growth of inoculated plants as "key" attributes. Several physical, physiological or biochemical stress effectors have been studied and reported in the past for some *Azospirillum* strains. However, no references are available in relation to the light effects on these bacteria. Our results showed that day light is a powerful effector with capacity to modify the bacterial physiology and behavior under planktonic culture conditions. Then, this effector should be considered in the future to improve both the production of biofertilizers and the selection of new strains of *Azospirillum*.

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Conflicts of interest

The authors report no conflicts of interest.

Author contribution

Romina Molina, Gastón López, Anahí Coniglio and Ana Furlan (1) Substantial contribution to conception and design or the acquisition and analysis of data. Verónica Mora, Susana Rosas and Fabricio Cassán (2) Drafting or critically revising the manuscript. Fabricio Cassán (3) Approval of the final submitted version.

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Table Header

Table 1 IAA concentration (μg ml^{-1}) and *ipdC* gene expression (Miller Units) by *A. brasilense* Az39 pFAJ64 in Luria Bertani liquid culture medium exposed to different conditions of white light (DL); blue (BL); red (RL) or

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darkness (D) after 24, 48 and 72 h of incubation at 36ºC. The values correspond to the mean ± the standard deviation. The same letters indicate that there are no significant differences between treatments, according to Tukey Test ($P < 0.05$)

	24 h		**48 h**		**72 h**	
	IAA ($\mu g\ ml^{-1}$)	***ipdC* expression (Miller Units)**	**IAA ($\mu g\ ml^{-1}$)**	***ipdC* expression (Miller Units)**	**IAA ($\mu g\ ml^{-1}$)**	***ipdC* expression (Miller Units)**
DL	16.31^{b}	220.87^{b}	33.27^{a}	408.82^{a}	30.37^{a}	413.99^{a}
BL	13.51^{c}	185.61^{cd}	17.03^{b}	191.66^{cd}	6.92^{f}	193.55^{c}
RL	7.94^{f}	111.48^{f}	10.57^{d}	138.34^{e}	14.62^{b}	186.77^{cd}
D	9.22^{e}	128.92^{e}	15.74^{b}	190.08^{cd}	15.64^{b}	193.12^{c}

Legend to Figures

Figure 1 Biomass production (OD_{595}) in *A. brasilense* Az39 cultures obtained in Luria Bertani liquid medium subjected to different light conditions: white light or DL (●) (1a); blue or BL (▼)(1b); red or RL (▲)(1c), compared to the control kept in dark conditions or D (■) during 24, 48 and 72 h of incubation at 36ºC. The symbols correspond to the mean ± the standard deviation. The same letters indicate that there are no significant differences between treatments, according to Tukey Test ($P < 0.05$)

Figure 2 Aggregation percentage (% AP) by *A. brasilense* Az39 in Luria Bertani liquid culture medium exposed to different conditions of white light or DL (●); blue or BL (▼); red or RL (▲) and darkness or D (■), after 24, 48 and 72 h of incubation at 36ºC. The symbols correspond to the mean ± the standard deviation. The same letters indicate that there are no significant differences between treatments, according to Tukey Test ($P < 0.05$)

Figure 3 Production of biofilms (OD_{560}) by *A. brasilense* Az39 in Luria Bertani liquid culture medium exposed to different conditions of white light or DL (●); blue or BL (▼); red or RL (▲) and darkness or D (■), after 24, 48 and 72 h of incubation at 36ºC. The symbols correspond to the mean ± the standard deviation. The same letters indicate that there are no significant differences between treatments, according to Tukey Test ($P < 0.05$)

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Figure 4 (a) Hydrogen peroxide content (nmol H_2O_2 mg^{-1}protein) and (b) TBARs (nmol TBARs mg^{-1} protein) in cultures of *A. brasilense* Az39 in Luria Bertani liquid medium exposed to different light conditions: white light (DL); blue (BL); red (RL) and darkness (D), after 24 h incubation at 36ºC. The bars correspond to the mean ± the standard deviation. The same letters indicate that there are no significant differences between treatments, according to Tukey Test ($P < 0.05$)

Figure 5 Activities of the enzymes (a) CAT (μmol H_2O_2 $min^{-1}mg^{-1}$ protein) and (b) SOD (U mg^{-1} protein) in cultures of *A. brasilense* Az39 in Luria Bertani liquid medium exposed to different light conditions: white light (DL); blue (BL); red (RL) and darkness (D), after 24 h incubation at 36ºC. The bars correspond to the mean ± the standard deviation. The same letters indicate that there are no significant differences between treatments, according to Tukey Test ($P < 0.05$)

Supplementary material

Figure S1 Cell viability (log10 CFU ml^{-1}) in cultures of *A. brasilense* Az39 in Luria Bertani liquid medium subjected to different light conditions: daylight or DL (●) (1a); blue or BL (▼) and red light or RL (▲) compared to the control kept in dark conditions or D (■) during 24, 48 and 72 h of incubation at 36ºC. The symbols correspond to the mean ± the standard deviation. The same letters indicate that there are no significant differences between treatments, according to Tukey Test ($P < 0.05$)

Figure S2 Indole-3-acetic acid concentration (IAA μg ml^{-1}) in Luria Bertani liquid medium subjected to different light conditions: daylight or DL (●) and blue light or BL (▼) compared to the control kept in dark conditions or D (■) during 24, 48 and 72 h of incubation at 36ºC. The symbols correspond to the mean ± the standard deviation. The same letters indicate that there are no significant differences between treatments, according to Tukey Test ($P < 0.05$)

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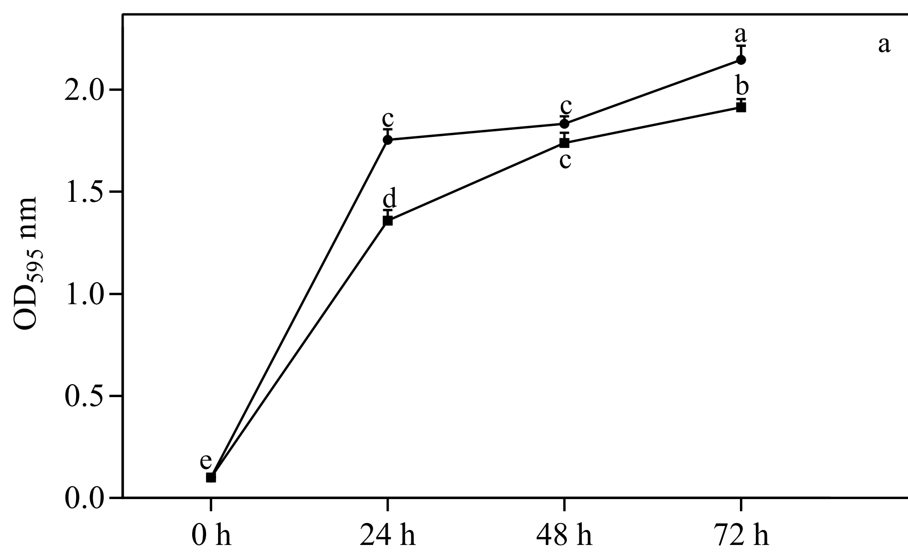
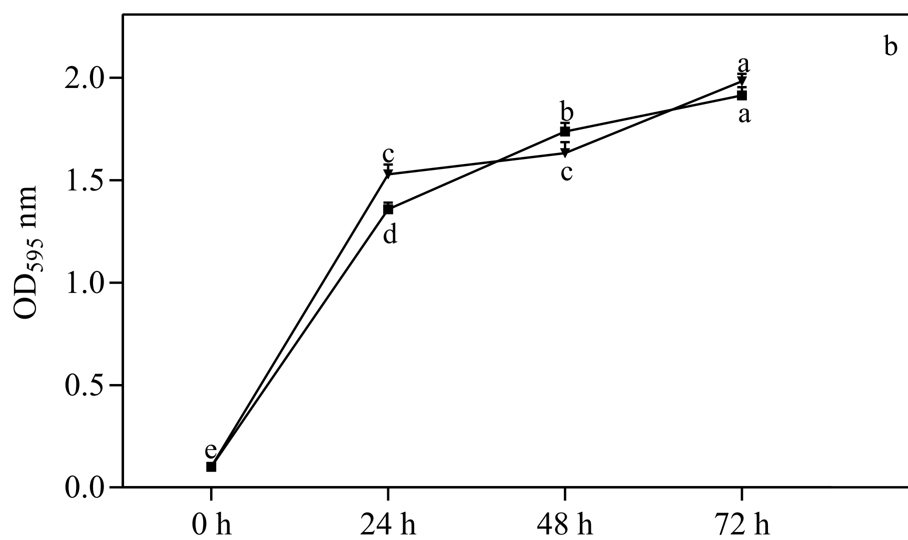
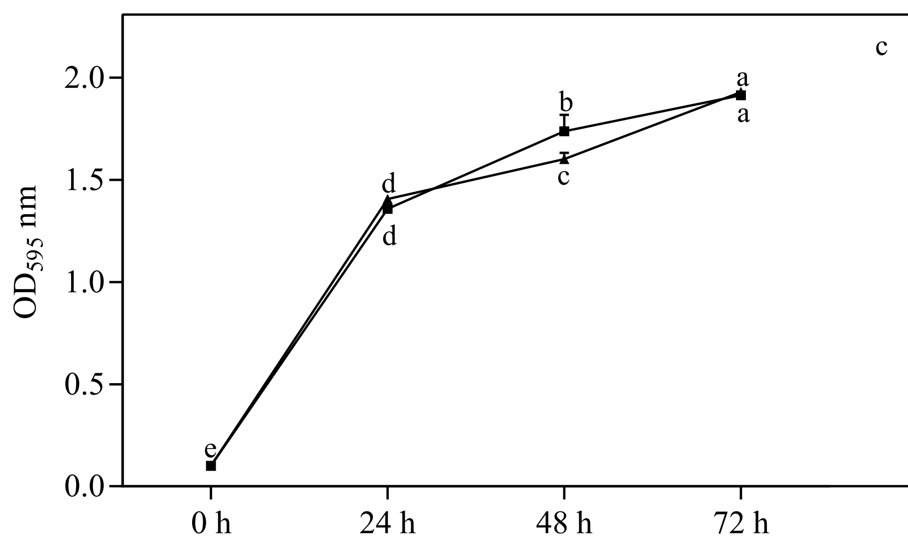
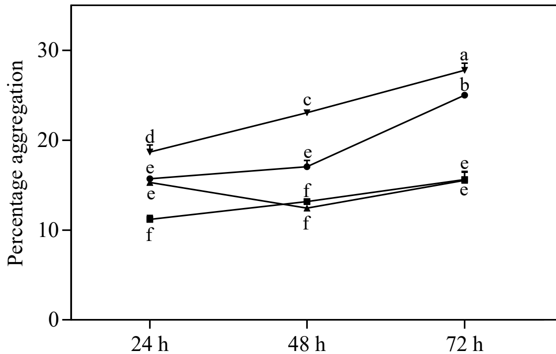
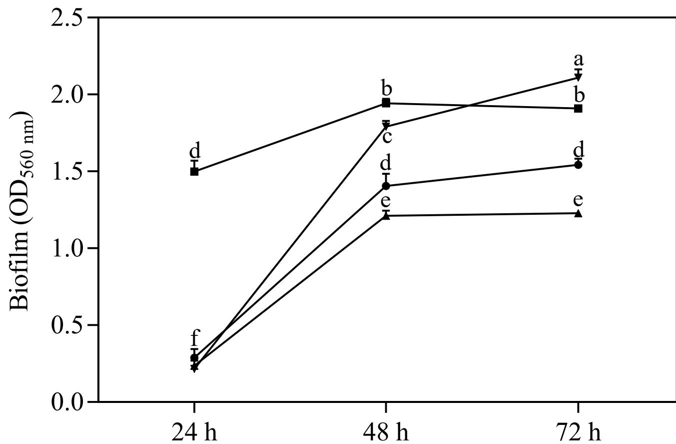
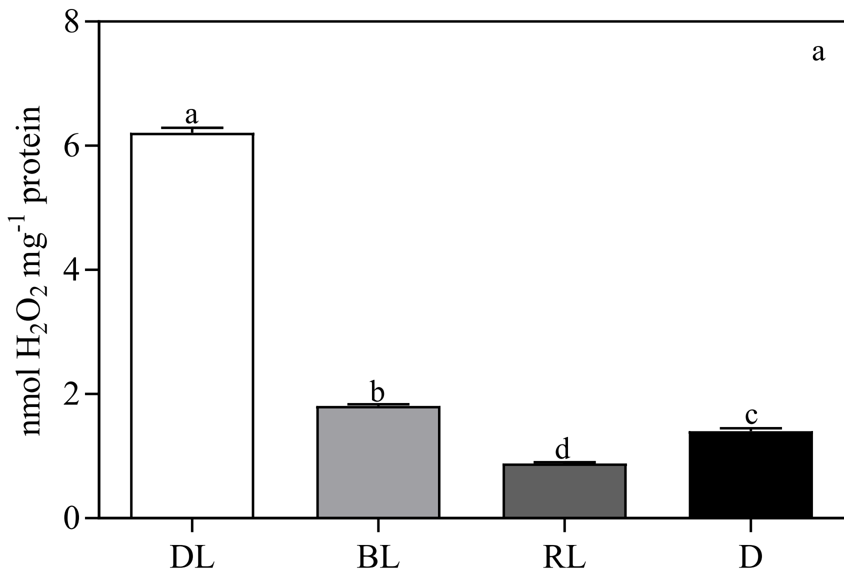

a
OD595 nm
0.0
0.5
1.0
1.5
2.0
0 h
24 h
48 h
72 h
a
b
c
c
c
d
e


b
OD595 nm
0.0
0.5
1.0
1.5
2.0
0 h
24 h
48 h
72 h
a
a
b
c
c
d
e

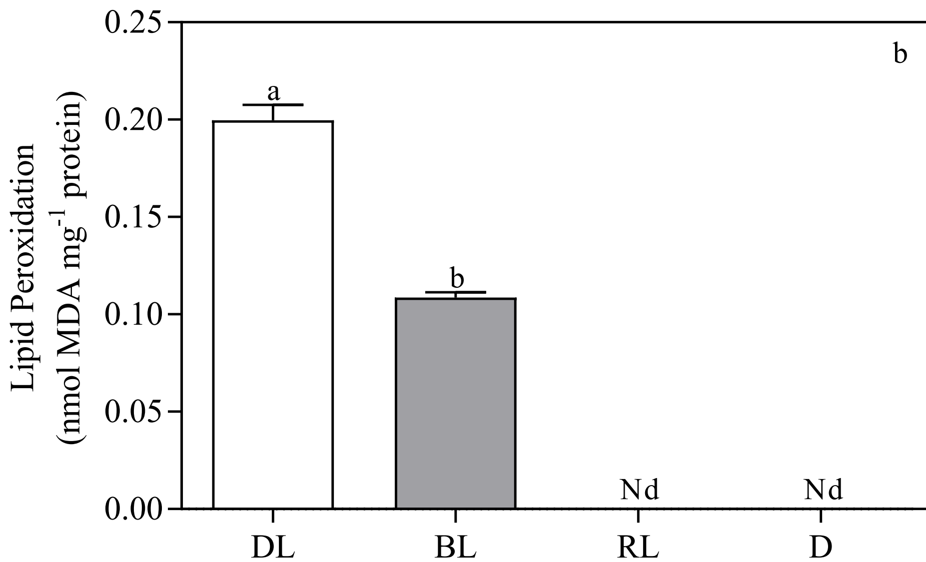

c
OD595 nm
0.0
0.5
1.0
1.5
2.0
0 h
24 h
48 h
72 h
a
a
b
c
d
d
e


Percentage aggregation
30
20
10
0
24 h
48 h
72 h
a
b
c
d
e
e
e
e
f
f
f
e
e

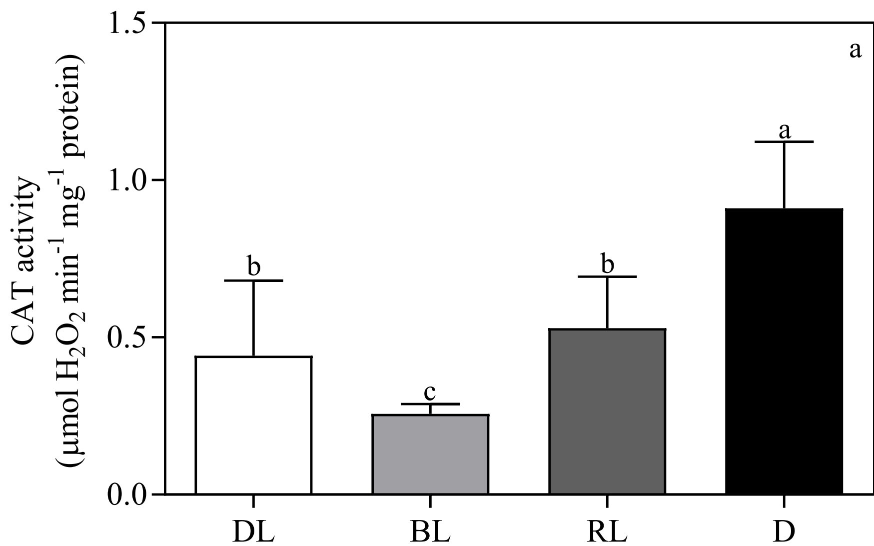

Biofilm ($OD_{560\ nm}$)
2.5
2.0
1.5
1.0
0.5
0.0
24 h
48 h
72 h
a
b
b
d
c
d
d
e
e
d
f



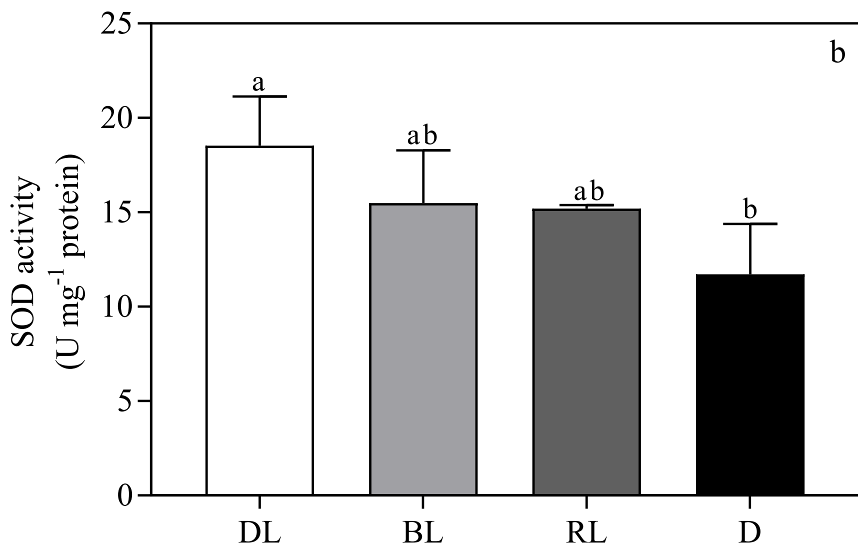
nmol H_2O_2 mg^{-1} protein
8
6
4
2
0
a
a
b
d
c
DL
BL
RL
D



Lipid Peroxidation
(nmol MDA mg^{-1} protein)
0.25
0.20
0.15
0.10
0.05
0.00
b
a
b
Nd
Nd
DL
BL
RL
D



CAT activity
(μmol H_2O_2 min^{-1} mg^{-1} protein)
1.5
1.0
0.5
0.0
a
b
c
b
a
DL
BL
RL
D



SOD activity
(U mg^{-1} protein)
25
20
15
10
5
0
b
a
ab
ab
b
DL
BL
RL
D