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Maize drought protection by *Azospirillum argentinense* Az19 requires bacterial trehalose accumulation

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Abstract

Azospirillum argentinense Az19 is an osmotolerant plant growth-promoting bacterium that protects maize plants from drought. In this work, we explored the role of trehalose in the superior performance of Az19 under stress. The trehalase-coding gene *treF* was constitutively expressed in Az19 through a miniTn*7* system. The resulting recombinant strain, Az19F, did not accumulate trehalose, was affected in its capacity to cope with salt-, osmotic-, and UV-stress, and showed higher reactive oxygen species levels. Physiological alterations were also observed under normal conditions, such as increased growth in biofilms, higher motility, and decreased auxin secretion. Even so, the capacity of Az19F to colonize maize roots was not affected, either under normal or drought conditions. When inoculated in maize, both Az19 and Az19F strains promoted plant growth similarly under normal irrigation. However, unlike Az19, the trehalose-deficient strain Az19F could not improve the height, aerial fresh weight, or relative water content of maize plants under drought. Notably, Az19F triggered an exacerbated oxidative response in the plants, resulting in higher levels of antioxidant and phenolic compounds. We conclude that the role of trehalose metabolism in *A. argentinense* Az19 transcends stress tolerance, being also important for normal bacterial physiology and its plant growth-promoting activity under drought.

Key points

- *Trehalose is required by Az19 for full tolerance to salt-, osmotic-, and UV-stress.*
- *A restriction in trehalose accumulation alters Az19 normal cell physiology.*
- *Trehalose contributes to Az19-induced maize growth promotion under drought.*

Keywords PGPR · Abiotic stress · Osmolyte · Inoculant · Biofertilizer

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Introduction

Plant-associated bacteria belonging to the *Azospirillum* genus are of great agricultural importance due to their remarkable abilities to improve the growth and yield of various crops. To harness this biotechnological potential, several *Azospirillum* strains have been included in formulates that are used in agriculture as inoculants. Over the years, extensive studies have elucidated that azospirilla exerts plant growth-promotion (PGP) through multiple mechanisms, of which nitrogen fixation and the production of indole-3-acetic acid (IAA) are the two best studied ones (Cassán et al. 2020). In addition, some strains are able to improve plant tolerance to abiotic stresses, including drought, salinity, heat, and heavy metals contamination (Cruz-Hernandez et al. 2022; Fukami et al. 2018a). Several mechanisms by which azospirilla can ameliorate plant stress have been proposed:

the synthesis and/or manipulation of stress-signaling growth regulators such as abscisic acid, ethylene, polyamines and nitric oxide (Cassan et al. 2009; Cohen et al. 2015; Creus et al. 2005; Degon et al. 2023; Perrig et al. 2007). However, direct evidence of *Azospirillum*-mediated plant protection by these mechanisms is still limited in most cases.

A common strategy of bacteria to cope with osmotic stress is the accumulation of protective molecules generically termed osmolytes or compatible solutes. These small organic compounds of different chemical nature can protect cell structures from stress by maintaining the cell turgor pressure and the integrity of cellular structures and molecules (Gregory and Boyd 2021). One of the best known osmolytes is the disaccharide trehalose, which can function as an osmoprotectant, a chaperone, be a carbon source, regulate cell metabolism, scavenge free radicals, or intervene in plant-pathogen interactions (Kuczynska-Wisnik et al. 2024). Trehalose is a key player in stress tolerance of several bacterial groups (Vanaporn and Titball 2020). However, in the case of *Azospirillum*, the role of this molecule in stress response is controversial. Madkour et al. (1990) were unable to detect trehalose in cells of different *Azospirillum brasilense* strains under high osmolarity while, on the other hand, Hartmann et al. (1991) found that this osmolyte is important for the adaptation of *A. brasilense* Sp7 and *Azospirillum halopraeferens* Au4 to high salinity.

Azospirillum argentinense Az19 is an osmotolerant strain that is able to ameliorate the negative effects of drought in maize, both under controlled and field conditions (García et al. 2017, 2023). The sequencing of Az19 genome revealed previously unattended characteristics that suggest that this strain might be adapted to survive in the phyllosphere (García et al. 2020). Although the genetic content of Az19 potentially codes for several stress-coping mechanisms that could explain its endurance and plant-protective effect under stress, the precise physiological basis of this characteristic has not been established yet. Interestingly, García et al. (2017) detected a strong induction of trehalose production in strain Az19, 388% higher than the one experimented by the reference strain *A. argentinense* Az39. This evidence suggested a possible link between trehalose accumulation and Az19 resilience under osmotic stress. Indeed, it has been proven before that trehalose overproduction in *A. argentinense* improves bacterial tolerance to osmotic stress and results in maize protection from drought after inoculation (Rodríguez Salazar et al. 2009).

This work delves into the importance of trehalose accumulation on *A. argentinense* Az19 stress management. By following a trehalose-degradation strategy, we aimed to obtain direct evidence of the specific role of this compound on Az19 endurance against abiotic stress and on the inoculation-mediated protection of maize when subjected to drought.

Materials and methods

Bacterial growth and recombinant strains construction

A. argentinense Az19 (formerly *A. brasilense* Az19) is deposited in the BPCV Collection (World Federation for Culture Collection-WDCM31) under access number LBPCV19. Strain information and culture conditions have been detailed previously (García et al. 2017). A miniTn*7* transposon that contains gentamicin resistance and *treF*-expression cassettes was obtained by inserting the *treF* coding sequence from *Escherichia coli* K12 (Accession NC_000913.3, locus tag b3519). To this end, the *treF* coding sequence was amplified by PCR using primers Ec.K-RBS-TreF FW (GGTACCTTTAAGAAGGAGATATCA TGCTCAATCAGAAAATTCA) and Ec.H-TreF RV (AAG CTTATGGTTCGCCGTACAAACC), purified and cloned into pGemT-Easy (Promega, Madison, USA). The insert, which included an upstream ribosome binding site, was then transferred to the miniTn*7* plasmid pMT7Ga (Labarthe et al. 2024), downstream of the $P_{A1/O4/O3}$ promoter, using *Eco*RI and *Spe*I restriction enzymes. The resulting plasmid pMT7Ga-R-treF was introduced into strain Az19 for the integration of engineered transposon into the chromosome, as described before (Maroniche et al. 2018). The control recombinant strain Az19gm used in this work, which carries the miniTn*7* transposon without the *treF* expression cassette, was obtained by the same procedure using the parental pMT7Ga plasmid. The correct insertion of the transposon, downstream of the *glmS* gene, was confirmed by PCR using primers Az-GlmS FW (5'-CCCCGCTGCTCTACGCCATC-3') and Tn*7*L RV (5'-ATGGGAACTGGGTGTAGCGT-3'), and the amplification product was sequenced to determine the exact site of chromosomal integration. All the recombinant strains were routinely cultured on RC medium supplemented with 10 µg/mL of gentamicin.

Fluorescent variant strains expressing DsRed were obtained by introducing the plasmid pME7134mob through triparental mating, as explained elsewhere (Maroniche et al. 2018).

Trehalose detection

Intracellular trehalose production was analyzed by thin layer chromatography (TLC). *A. argentinense* Az19gm and Az19F were cultured in 5 mL of NYA (García et al. 2017), with or without 300 mM NaCl, for 48 h at 28 °C. The cells were recovered by centrifugation at 13,000 × *g* and 4 °C for 2 min, and washed with ultrapure water. After

a second centrifugation step, cells were suspended in 200 µL of ultrapure water before adding 800 µL of ethanol. Cells in 80% ethanol were lysed by incubating at 85 °C for 15 min and then centrifuged at 13,000 × *g* for 15 min. The supernatant (950 µL) was transferred to a new tube and completely evaporated at 85 °C. The dry extract was resuspended in 100 µL of ultrapure water and stored at −80 °C until analyzed by thin layer chromatography (TLC). The extracts were spotted (2 µl) in a silica gel 60 aluminium sheet (Merck, Darmstadt, Germany), resolved with an n-butanol:pyridine:water (7:3:1) mobile phase, and revealed with 20% H_2SO_4 in ethanol and heating at 120 °C for 10 min. Glucose (20 mg/mL), sucrose, maltose, and trehalose (10 mg/mL) standards were included as references.

Stress tolerance assays

Bacterial tolerance to 200 mM NaCl, 20% polyethylene glycol (PEG), and 200 J/m^2 UV-C was evaluated as detailed by García et al. (2017) and García et al. (2020), respectively, with 3 independent replicates (cultures). Survival percentages were calculated for each replicate as the ratio of colony forming units (CFU) counted in stress vs control conditions. The assays were repeated at least 3 times.

To analyze extreme temperature tolerance, bacterial suspensions (4 independent replicates) were obtained from cultures in nutrient broth (NB, Laboratorios Britania, Buenos Aires, Argentina) by centrifugating and adjusting to $OD_{600nm} = 1$ with sterile saline solution (SS). The initial number of CFU in the suspension was determined by the drop plate method (Di Salvo et al. 2022). For heat-shock treatments, 0.5 mL of the suspensions was transferred to 1.5-mL microcentrifuge tubes, incubated at 55 °C for 30 min and then used to count the number of surviving CFU in each tube. For freezing treatment, 0.5 mL of bacterial suspensions were frozen at −20 °C for 10 days, and then thawed for CFU count. The count values obtained after stress treatments were relativized to control conditions and expressed as percentage of surviving CFU.

Reactive oxygen species (ROS) production was evaluated with the probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen, Waltham, USA). A 10 mM H_2DCFDA stock solution was prepared in dimethyl sulfoxide and used to prepare a 12 µM working solution by diluting in PBS. Bacteria were cultured in NYA at 28 °C and 150 rpm for 48 h, centrifuged and resuspended in sterile ultrapure water at $OD_{600nm} = 2$. Each suspension was immediately mixed (2:1) with the probe inside a well of a 96-well microplate in a final volume of 300 µL and incubated at 37 °C for 2 h. Then, the green fluorescence (485/525 nm excitation/emission) was measured with a microplate reader (FLx800, BioTek Instruments, Winooski, USA). Bacterial CFU present in the suspensions were determined and used to relativize the fluorescence values.

Analysis of *in vitro* PGP characteristics

Biofilm production was initially analyzed by the crystal violet method as described by Maroniche et al. (2024). Then, a scaled-up method was developed to analyze cell population and exopolysaccharides (EPS) production in static-culture biofilms. Bacterial suspensions obtained from overnight cultures of Az19gm-DsRed and Az19F-DsRed in NB were adjusted to $OD_{600nm} = 1$, mixed 1/100 with fresh medium (NYA or NYA plus 300 mM NaCl) and pipetted into 90 mm Petri dishes (15 mL per plate) to initiate biofilms. After 48 h of incubation at 28 °C under static conditions, the supernatant was carefully recovered to analyze planktonic cell number, and the remaining cells adhered to the plate were suspended in 15 mL of SS after mechanically disaggregating the biofilm with a pipette tip. The red fluorescence of the resulting suspensions was measured with a microplate reader (FLx800, BioTek Instruments, Winooski, USA). In addition, CFU count and fluorescence of ½ serial dilutions (up to 1/8) were analyzed in only one replicate of each strain/condition, to obtain a fluorescence/CFU calibration curve. This calibration was used to calculate CFU values for the rest of the replicates. A second set of plates was used for EPS extraction. To this end, the whole content of each plate was recovered and treated with NaCl in a final concentration of 1 M to release the EPS attached to cells (Chiba et al. 2015). Samples were centrifuged for 15 min at 3000 × *g* and 4 °C to pellet the cells, the supernatant was transferred to a new tube and EPS were precipitated with 2 vol of ethanol. After a 3-day incubation at −20 °C, polysaccharides were pelleted by centrifugation at 3000 × *g* and 4 °C for 15 min, dried at room temperature and resuspended in 1 mL of ultrapure water. EPS concentration in the extracts was quantified by the phenol–sulfuric method (DuBois et al. 1956).

Swimming assay consisted in inoculating bacterial suspensions adjusted to an optical density at 600 nm (DO_{600nm}) of 1 into the center of plates with NYA semisolid medium (0.3% agar), using a sterile toothpick. The plates were incubated at 28 °C for 72 h, and then photographed in a UV-transilluminator (ImageQuant 300, GE Healthcare, Chicago, USA).

To estimate auxins production, indolic compounds were quantified by the Salkowski method (Glickmann and Dessaux 1995) using the supernatant of cultures grown in NYA + 1 g/L tryptophan, and incubated at 28 °C with 150 rpm shaking for 48 h. Relative auxin concentrations were estimated by extrapolation to a standard curve of IAA and subsequent relativization to the OD_{600nm} of the cultures.

Maize inoculation trial and bacterial count in roots

Plant assays were done essentially as described by García et al. (2017). Briefly, maize seeds (hybrid DOW 510 PW) were superficially sterilized, pre-germinated, and inoculated with Az19 or Az19F (final dose per seed: 5×10^7 CFU), or not inoculated. The treated seeds were sown in 0.75 L pots filled with a mixture of sand:subtrate:vermiculite:perlite (3:3:3:1), which were previously watered to full (control treatments) or 50% field capacity (drought treatments). Plants were incubated in a growth chamber at 26 °C under a 16/8 h of light/darkness photoperiod, and irrigated when needed (control), or not watered (drought). At day 15, plants were harvested to measure growth parameters and relative water content (RWC).

To analyze bacterial root colonization and survival, maize seeds were inoculated as described before. A set of seeds were used 2 h post-inoculation (hpi) to analyze the initial number of established bacteria. To this end, 50 seeds per treatment were soaked in 50 mL of SS for 15 min, and then serially diluted for subsequent CFU count on RC medium supplemented with 10 µg/mL gentamicin. The rest of the seeds were sown and subjected to control or drought conditions as explained above. At 7 days post-inoculation (dpi), roots were extracted, cleaned carefully to remove the adhered substrate, and ground in a mortar with 100 mL of SS. The resulting homogenates were serially diluted in SS and used for CFU count.

Biochemical analyses of plant material

After carrying out a maize inoculation assay, as described above, the last completely expanded leaves of 3 plants per treatment were collected, immediately freezed in N_2 and freeze-dried until their analysis. To determine the content of phenolic compounds, 150 mg of lyophilized tissue were extracted with 3.75 mL of ethanol acidified with 50 µl of HCl, and analyzed as detailed by Moreno-Escamilla et al. (2015). To determine the antioxidant capacity, an extract of 150 mg of tissue in 1.5 mL of ethanol was analysed by the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) methods (Stratil et al. 2006; Lee et al. 2015).

Experimental design and statistical analyses

All experiments were carried out with at least three independent replicates. Plant inoculation assays were distributed in a completely randomized design, with 20 plants (replicates) for each of the treatments that resulted from the combination of two factors: inoculum (3 levels: Az19, Az19F, and non-inoculated control) and stress (2 levels: normal irrigation and water restriction). Data was statistically analyzed with Prism 9 (GraphPad Software Inc., San Diego, USA) as detailed in the legend of each figure. Plots depict means plus standard deviation. Differences were considered to be significant at $p \leq 0.05$. When required, data was transformed to meet the statistical tests criteria.

Results

TreF expression in *A. argentinense* Az19 prevents trehalose accumulation

A. brasilense Az19 was modified to inactivate intracellular trehalose accumulation by the heterologous expression of TreF, the cytoplasmic trehalase of *E. coli* (Horlacher et al. 1996). A transposon containing gentamicin-resistance and *treF*-expression cassettes (Fig. 1A) was inserted into the chromosome Az19 using the miniTn*7* system (Koch et al. 2001). Three independent clones (C1, C2, and C3) were analyzed by PCR-amplification and sequencing to confirm the correct insertion of the transposon (Fig. 1B). The PCR product was sequenced to determine the exact insertion site of the Tn*7* transposon (Fig. 1C). The recombinant clone C3, termed Az19F from now on, was selected for further experiments. Another recombinant strain carrying the empty miniTn*7* transposon was constructed to serve as the control strain (Az19gm).

To characterize the phenotype of Az19F, conditions for the induction of trehalose synthesis by NaCl were first assessed in Az19gm (Fig. 1C). Trehalose production was readily visualized after 24 h of growth in NYA medium amended with 150 mM NaCl, and increased with time and a higher NaCl concentration in the medium (Fig. 1D). According to these results, Az19F was cultivated for 48 h with 300 mM NaCl to confirm the degradation of intracellular trehalose levels due to TreF activity (Fig. 1E).

Trehalose accumulation contributes to Az19 abiotic stress tolerance *in vitro*

The role of trehalose production in *A. argentinense* Az19 abiotic stress tolerance was investigated by measuring bacterial survival in the presence of the stressor in comparison to control conditions. The results showed that Az19F has a lower capacity than the control strain Az19gm to cope with salinity, as revealed by the reduced number of surviving cells (Fig. 2A) and strong aggregation (Fig. 2B) when cultured with 200 mM NaCl. Az19F was also more sensitive to osmotic stress induced by PEG (Fig. 2C) and to UVC exposure (Fig. 2D). Moreover, colonies formed on RC medium by the surviving cells of Az19F after UV treatment were small and unstained, in contrast to Az19gm which developed normal-sized red colonies (Fig. 2E). On the other hand, the

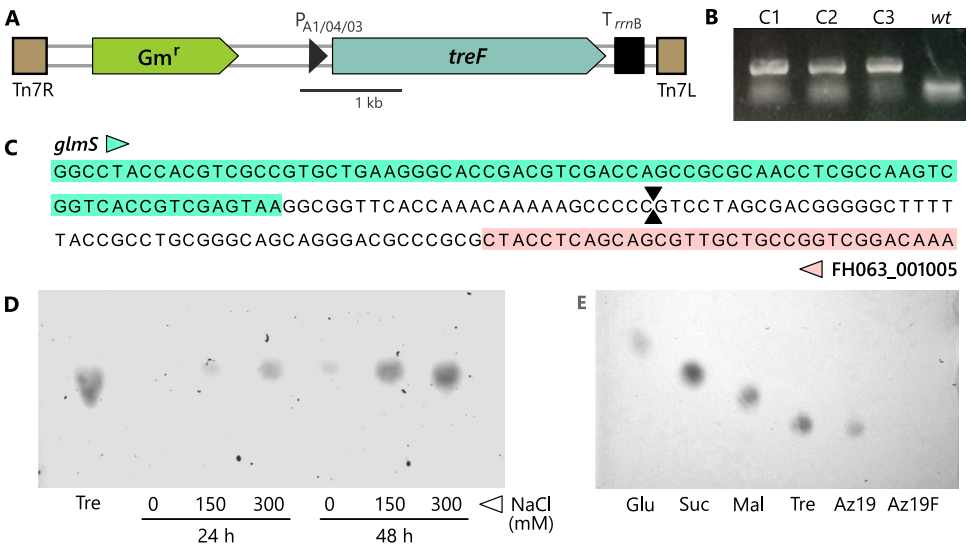

Fig. 1 Construction of the trehalose-depleted recombinant strain *A. argentinense* Az19F. **A** Schematic representation of the constructed miniTn*7*, carrying gentamicin resistance (Gm^r) and *treF*-expression cassettes. **B** PCR amplification products confirming the correct insertion of the miniTn*7* in the chromosome of the recombinant clones C1, C2 and C3, and its absence in Az19 (*wt*). **C** Genomic region downstream of *glmS* gene, where the miniTn*7* was inserted (black arrows) **D** Screening of conditions for NaCl-induced trehalose accumulation in *A. argentinense* Az19 by TLC. Three NaCl concentrations (0, 150 and 300 mM) and two induction times (24 and 48 h) were tested. A trehalose standard was used as reference (Tre). **E** Confirmation of trehalose degradation in the mutant strain Az19F by TLC. Glucose (Glu), sucrose (Suc), maltose (Mal) and trehalose (Tre) standards were used as references

trehalose-deficient strain Az19F showed the same capacity to tolerate a heat shock (Fig. 2F) or freezing (Fig. 2G) as the control strain Az19gm.

For a more general characterization of trehalose effect in the bacterial oxidative status, intracellular ROS content was measured in Az19gm and Az19F cells using the probe H_2DCFDA. Notably, ROS levels in Az19F were higher, not only under salinity but also in control conditions (Fig. 2H).

Trehalose degradation affects Az19 *in vitro* traits associated to biostimulation, but does not alter root colonization

The production of biofilm and phytohormones, as well as mobility, are important physiological characteristics of plant-associated beneficial bacteria involved in root colonization and PGP. We evaluated if *A. argentinense* Az19F defect in trehalose accumulation alters PGP-associated characteristics such as biofilm formation, swimming, auxins secretion and maize root colonization.

Upon assessing biofilm formation by the classic crystal violet method, it was determined that the mutant strain Az19F doubles the production of biofilm of strain Az19gm under control conditions. However, this strain suffers a significant reduction of biofilm production under salt stress conditions, while no difference is observed for Az19gm between control and salinity conditions (Fig. 3A). Further experimentation was done to determine if the increase in Az19F biofilm in control conditions is due to an enhancement in either growth or biofilm matrix production. The results revealed that Az19F growth in static cultures under control conditions is boosted, resulting in a higher population of planktonic cells (Fig. 3B). However, under salinity, and unlike Az19gm, Az19F cell count decreased when compared to control conditions. This decrease in Az19F growth under salinity was accompanied by a sharp increase in EPS production, which was higher than that of the control strain Az19 (Fig. 3C).

In agreement with the higher planktonic cell population of Az19F, swimming assays revealed that this strain is hypermobile under control conditions, producing a significantly bigger ($p < 0.0001$) swimming halo than the control strain Az19gm. On salt stress conditions, bacterial swimming was impaired in both strains (Fig. 3D).

The production of auxins was reduced by approximately 50% under control conditions in the trehalose-deficient strain Az19F when compared to Az19gm (Fig. 3A). This deficiency was not observed under salinity (Fig. 3E).

To evaluate root colonization capacity, maize seeds were inoculated with Az19gm or Az19F and sown. The resulting plants were raised under normal irrigation or drought, and their roots were analyzed for bacterial colonization at 7 dpi. The results showed no difference in the capacity of Az19 and Az19F to establish on the seeds and subsequently colonize the roots of the developing plants, both strains being similarly affected by drought ($p = 0.009$) (Fig. 3F).

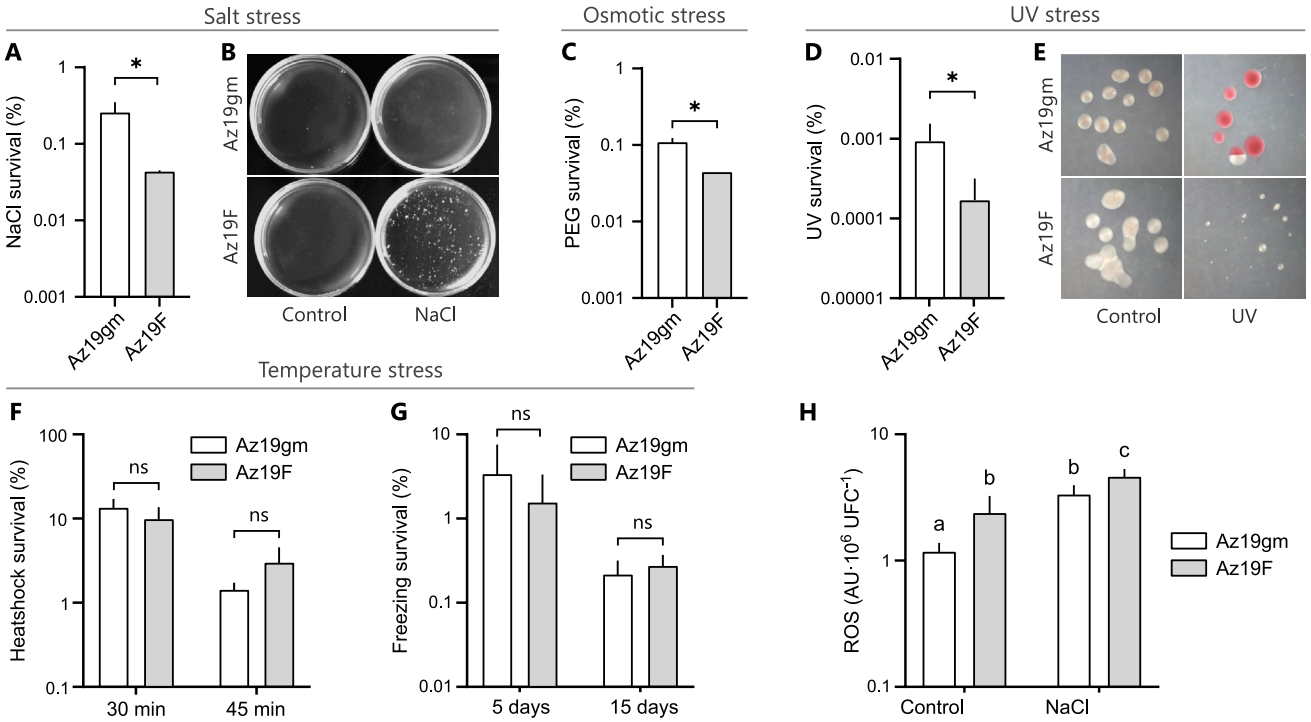

Fig. 2 Effect of trehalose degradation on Az19 stress tolerance. **A** Salinity tolerance was evaluated as the bacterial survival after 48 h of incubation in NYA medium containing 200 mM NaCl. Survival was estimated as the percentual relation between the final and initial CFU number. **B** Representative photographs of 35 mm Petri dishes containing bacterial cultures after incubation in normal conditions or salt stress. **C** Tolerance to 20% PEG after 48 h of growth in NYA medium. **D** Tolerance to UV stress was estimated as the percentage of surviving CFU that grew on RC plates after irradiating with UVC light, in comparison to non-treated plates. **E** Representative photographs of Az19gm and Az19F colonies formed after UV treatment. **F** Percentage of surviving cells after receiving a heat-shock at 50 °C for 30 or 45 min. **G** Percentage of surviving cells after being frozen at −20 °C for 5 or 15 days. **H** Quantification of Az19gm and Az19F intracellular ROS levels under control or salt-stress conditions, using the probe H_2DCFDA. Asterisks denote significant differences according to a paired t-test. Different letters in (H) denote significant differences according to two-way ANOVA plus Tukey's test ($p \leq 0.05$)

Trehalose degradation disturbs *A. argentinense* Az19 potential to improve maize drought tolerance

Inoculation assays were carried out to test the impact of trehalose degradation on *A. argentinense* Az19-mediated maize growth improvement under drought. As expected, the growth of maize plants inoculated with *A. argentinense* Az19 was enhanced under both control and drought conditions, showing increased height (Fig. 4A), fresh and dry weight of the shoot, and root biomass (Fig. 4B). The degradation of trehalose in strain Az19F did not affect its capacity to improve maize growth under control conditions (Fig. 4A and B). However, Az19F had a defective performance under drought when compared to Az19, as revealed by its reduced capacity to improve the height (Fig. 4A) and fresh weight (Fig. 4B) of the inoculated plants. Moreover, inoculation with the trehalose-deficient strain Az19F did not increase the RWC of maize plants grown under water restriction, as did Az19 treatment (Fig. 4C).

Maize plants were also analyzed biochemically to assess their oxidative status. It was determined that inoculation with the trehalose-deficient mutant strain Az19F, but not Az19, triggered a strong oxidative response in the plants under drought stress, resulting in higher levels of leaf phenolic compounds (Fig. 5A) and antioxidants (Fig. 5B). Under control conditions, inoculation treatments did not affect these parameters when compared to the not-inoculated group (Fig. 5).

Discussion

A. argentinense Az19 is a strain with a high potential to improve the productivity of crops under semi-arid conditions. When compared to other strains, such as the reference strain *A. argentinense* Az39 that is commonly used in inoculant formulations, Az19 is more osmotolerant and prone to improving the growth of maize under drought in the laboratory, greenhouse, and field (García et al. 2017, 2023). The finding that Az19 is capable of producing high amounts of trehalose in vitro when exposed to salinity (García et al.

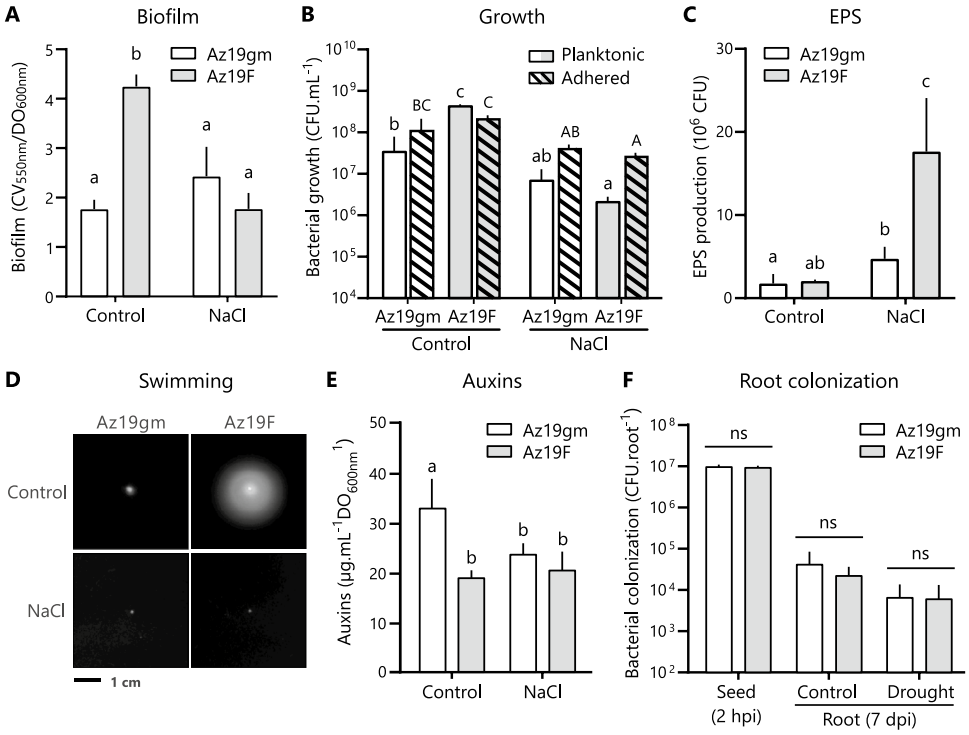

Fig. 3 Effect of trehalose degradation on Az19 physiological characteristics associated with plant growth-promotion. Characterization of Az19gm and Az19F PGP-related traits under salt stress (300 mM NaCl) or without it (Control). **A**, **B**, and **C** Strains Az19gm and Az19F were cultured statically in NYA medium to analyze biofilm production (A), planktonic and adherent growth (B), and EPS production (C). **D** Swimming mobility of Az19gm and Az19F in semisolid NYA medium. **E** Auxinic compounds secreted by Az19gm and A19F after 48 h of growth in NYA + tryptophan. **F** Az19 and Az19F recovery from maize seeds 2 h after inoculation (2 hpi), or from the roots after 7 days of growth (7 dpi) under normal irrigation or drought. In all cases, different letters indicate statistically significant differences according to two-way ANOVA plus Tukey's multiple comparison test ($p \leq 0.05$)

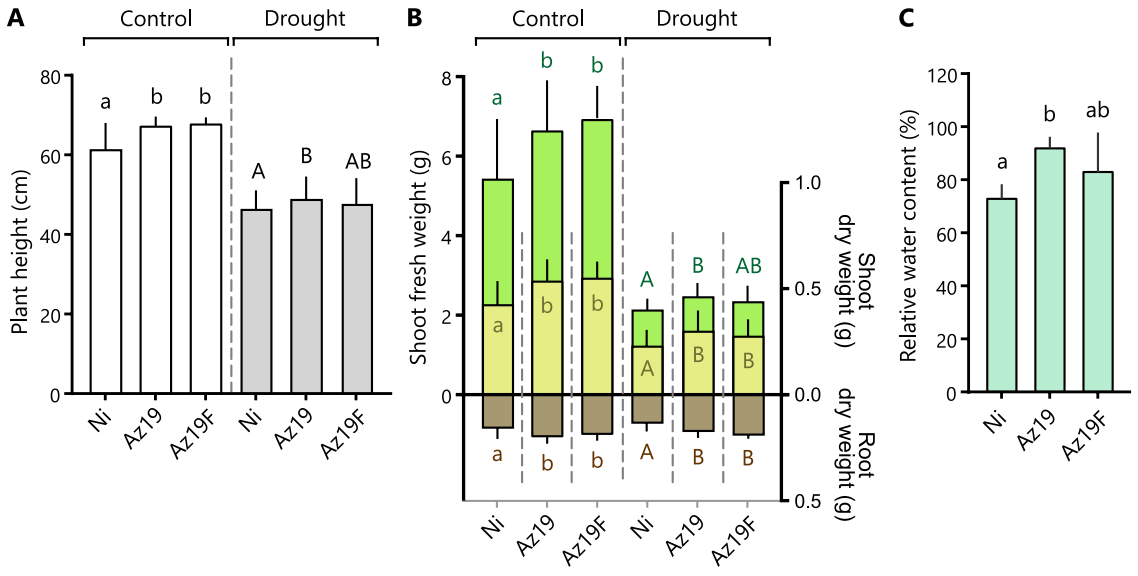

Fig. 4 Effect of trehalose degradation on Az19-induced maize drought tolerance. Maize seeds were inoculated with Az19 or Az19F, or not inoculated (Ni), sown in pots and incubated in a growth chamber under full (Control) or 50% field capacity (Drought). After 15 days, maize growth was analyzed by measuring plant height (**A**), fresh and dry weight of the shoot, dry weight of the root (**B**) and the leaf relative water content of stressed plants (**C**). Plots depict average values plus the standard deviation of three independent experiments. Data was analyzed by two-way ANOVA, plus Tukey's multiple comparison test ($p \leq 0.05$)

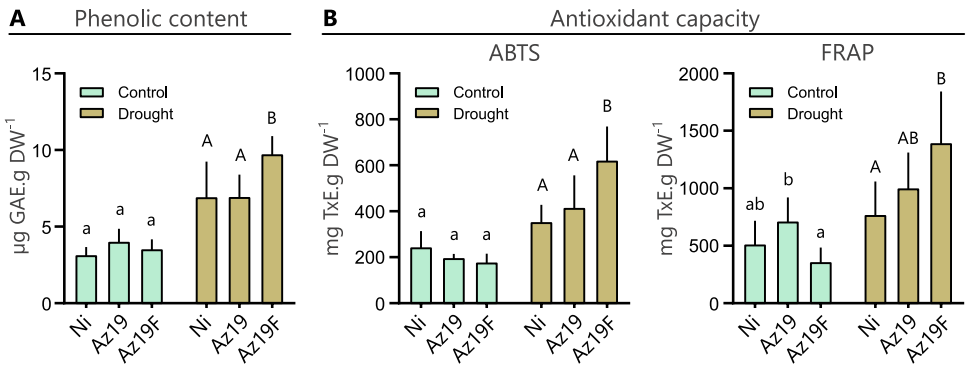

Fig. 5 Effect of Az19 trehalose degradation on the oxidative status of inoculated maize. Maize seeds were inoculated as described before, and the last completely expanded leaf of each plant was cut, immediately feezed in N_2 and kept at −80 °C until lyophilization. Samples were then processed to analyze total phenolic compounds (**A**) and antioxidant capacity by the ABTS and FRAP methods (**B**). Data was analyzed by two-way ANOVA, plus Tukey's multiple comparison test ($p \leq 0.05$)

2017) prompted us to investigate if this osmolyte contributes to the enhanced performance of Az19 under stress.

In bacteria, trehalose biosynthesis can be achieved through different biosynthetic pathways, being the OtsAB, TreYZ, and TreS pathways the most important (Vanaporn and Titball 2020). Although trehalose metabolism has not been specifically studied in *Azospirillum* yet, available genomic information suggests the existence these three main biosynthesis pathways (García et al. 2020). Thus, we opted to inactivate trehalose production in strain Az19 through the heterologous expression of the *E. coli* gene *treF,* which codes for a cytoplasmic trehalase (Horlacher et al. 1996). This strategy was recently proven to be as efficient as trehalose synthesis disruption to study the role of this osmolyte on *Bradyrhizobium* stress response (Ledermann et al. 2021). *A. argentinense* Az19 was successfully engineered with a miniTn*7* transposon to constitutively express the *treF* gene, obtaining recombinant clones that carry the transgene in the expected site of the chromosome and are unable to accumulate trehalose. These results reinforce our previous results (Maroniche et al. 2018; Pagnussat et al. 2023) about the usefulness of the miniTn*7* for *Azospirillum* genetic engineering, allowing a controlled and stable expression of foreign proteins from a single-copy chromosomal transgene, and thus avoiding instability issues associated with plasmids (Friehs 2004).

Previously, García et al. (2017) established that both *A. argentinense* Az39 and Az19 synthesize trehalose under salt stress. In this work, we went further to demonstrate, by comparing the survival rates of strains Az19 and the trehalose-defective mutant Az19F under different abiotic stresses, that trehalose production contributes to *A. argentinense* Az19 salt-, osmotic-, and UV-stress tolerance. The incapacity of Az19F to accumulate trehalose resulted in an increased cell susceptibility to stress, which was accompanied by higher intracellular ROS levels, suggesting that trehalose protects Az19 by mitigating the oxidative damage generated under stress. Protection was important even under control conditions, where ROS were probably produced due to the high oxygenation of the cultures. These results are in agreement with the ROS scavenging activity that has been proven for trehalose before (Oku et al. 2003). Our findings, on the other hand, demonstrate that trehalose is not required for heat or freeze tolerance in Az19, either because it is not produced under these conditions or by the existence of a more prominent protective mechanism in these cases.

This work revealed that trehalose degradation has far-reaching physiological consequences for *A. argentinense* Az19 other than stress susceptibility. When cultured statically in the absence of stress, Az19F produced significantly more biofilm and had exacerbated planktonic growth, probably due to its hyper-swimmer phenotype. Also, auxin production by Az19F was nearly halved under this condition when compared to the control strain Az19gm. These unexpected pleiotropic alterations suggest that trehalose metabolism may be important not only for stress endurance but also for regulating *Azospirillum* physiology during growth. A similar role for trehalose has been described in yeasts (Nwaka and Holzer 1998). In the light of this evidence, we hypothesize that, due to trehalose degradation by TreF and the consequent higher intracellular glucose pool, nutritional stress signaling at high Az19F population density fails, leading to exacerbated growth and motility under normal conditions. This hypothesis is supported by the lack of auxin synthesis induction, which is expected when the stationary phase is reached (Vande Broek et al. 2005). Concurrently, glucose excess could lead to the overproduction of exopolysaccharides and boost bacterial growth in biofilms, as was observed with the *treC* mutant of *Klebsiella pneumonia* (Wu et al. 2011). Under salinity, the physiological alterations of Az19F were withheld, resulting in a higher susceptibility to stress, as evidenced by a strong reduction of growth in

biofilms, a sharp increase in EPS production, the induction of a non-motile state, and equal auxin levels to those of the control strain Az19gm. Thus, under high salinity, the metabolism deregulation of Az19F seems to be overruled by a stress signaling pathway of higher order. In this context, the lack of trehalose-mediated protection may have reduced Az19F salt-stress tolerance.

None of the abovementioned changes altered Az19F root colonization capacity when compared to Az19gm, indicating that this process does not require trehalose production, either normally or under drought. This is in agreement with Rodriguez-Salazar et al. (2009), who observed that trehalose overproduction in *A. argentinense* Az39 does not alter its capacity to colonize maize roots. However, trehalose degradation did have a negative impact on *A. argentinense* Az19 biostimulatory potential when inoculated in maize, but only when subjected to drought. Plants raised under full field capacity irrigation benefited equally from both strains, increasing all the measured growth parameters to the same extent. Under drought, Az19F was unable to increase the "fresh" parameters (i.e., height and shoot fresh weight) and the relative water content of the leaves significantly, as did Az19. This partial loss of the bioprotective effect of Az19 upon trehalose degradation indicates that multiple mechanisms are acting in this strain to improve maize drought tolerance after inoculation.

Our results point to the metabolism of trehalose being required by Az19 to induce a stress response in the plant. However, it cannot be established if this effect is mediated by the direct action of bacterial trehalose, or by other factors whose induction is linked to this osmolyte's metabolism. For example, the defect in auxin production of Az19F could be impairing the induction of plant responses required for normal drought tolerance. Zhang et al. (2020) demonstrated that the exogenous application of IAA improves white clover drought tolerance, a response that involve changes in the levels of other plant hormones such as abscisic acid (ABA) and jasmonic acid. Indeed, the interplay between auxins and ABA to modulate plant-water status under drought is well documented (Sharma et al. 2023). However, spray-application of exogenous trehalose can also activate ABA signaling in plants (Ali and Ashraf 2011; Yu et al. 2019). Thus, it is possible that the secretion of auxins and/or trehalose (or a byproduct of it) by Az19 could be eliciting an ABA-dependent stress response, in turn inducing stomata closure to prevent water loss. Interestingly, *Azospirillum baldaniorum* Sp245 was shown to induce an ABA-mediated stress response in *Arabidopsis* (Cohen et al. 2015; Degon et al. 2023), although this response was attributed to the secretion of this plant regulator by the bacteria (Cohen et al. 2008). It would be interesting to investigate in the future how these phytohormones and trehalose-dependent effects interrelate to exert Az19-mediated drought stress protection on maize.

In opposition to previous reports about the induction of the plant antioxidant machinery under abiotic stress by *Azospirillum* inoculation (Checchio et al. 2021; El-Esawi et al. 2019; Fasciglione et al. 2015; Fukami et al. 2018b), we did not observe an increase of antioxidant and phenolic compounds in plants inoculated with the control strain Az19gm. Moreover, these compounds increased only in plants inoculated with the trehalose-depleted strain Az19F and subjected to drought. This unexpected effect may be associated with a defense response of the plants to the bacteria themselves; overproduction of flagellin, which is a known *A. argentinense* elicitor of plant defenses (Elías et al. 2022; Mora et al. 2023), or the higher levels of ROS observed in Az19F cells are possible explanations for this response.

In sum, we conclude that trehalose accumulation in *A. argentinense* Az19 not only contributes to bacterial survival under stress but also participates in the regulation of cell metabolism under normal conditions. When associated with maize plants, the inability to accumulate trehalose has no effect on Az19 root colonization and growth promotion under normal irrigation, but it does affect the bacterial biostimulation capacity under drought and elicits an exacerbated antioxidant response in the plants. Thus, trehalose metabolism is required by *A. argentinense* Az19 for a normal plant-bacteria mutualistic association under drought. Further research is required to unveil additional mechanisms that fully explain the stress-responsive characteristics of *A. argentinense* Az19.

Authors' contributions Conceptualization: GAM; Methodology: JEG, GAM; Formal analysis and investigation: JEG, LAP, MBA, EMC, PRD, MML, MVM, GAM; Writing—original draft preparation: GAM; Writing—review & editing: JEG, LAP, EMC, PRD, MML, MDG, GAM; Funding acquisition: JEG, CMC, GAM; Resources: JEG, CMC, GAM; Supervision: GAM.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval This article complies with ethical standards and does not contain any studies with human participants or animal performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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