Beneficial microbial species and metabolites alleviate soybean oxidative damage and increase grain yield during short dry spells

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ABSTRACT
Short dry spells are an important grain yield constraint in tropical regions. Plant growth-promoting bacteria (PGPB) and their metabolites can mitigate the impact of drought stress by promoting changes in plant metabolism, physiology, and biochemistry. However, the effects of PGPB on soybean [Glycine max (L.) Merril] under drought stress in tropical regions have not been established. The experiments were carried out under tropical field conditions with short dry spells. Therefore, in this study we used a three-factorial trial to evaluate the effects of bacterial consortium consisting of N2-fixing Bradyrhizobium japonicum (strain SEMIA 5079) and Bradyrhizobium diazoefficiens (strain SEMIA 5080), the biocontrol agent Bacillus subtilis (strain QST 713), and the plant growth-promoting Azospirillum brasilense (strains Ab-V5 and Ab-V6) with or without application of microbial secondary metabolites (MSM, rhizobial metabolites enriched in lipo-chitooligosaccharides (LCOs)) during two growing seasons. Photosynthetic pigments, gas exchange parameters, antioxidant enzyme activity and proline concentrations in leaves, nodulation, plant growth development and grain yield were evaluated. The bacterial consortium comprising Bradyrhizobium spp., A. brasilense strains and MSM application increased the contents of chlorophyll a (14.5 %), chlorophyll b (30.8 %), total chlorophyll (17.2 %), and total carotenoids (27.3 %) compared with Bradyrhizobium spp. treatment alone. This consortium also increased the net photosynthetic rate (17.7 %), stomatal conductance (56.5 %), internal CO2 concentration in the substomatal chamber (8.3 %), and transpiration (44 %) compared with plants that received the standard inoculation (Bradyrhizobium spp. only), while reducing the leaf contents of hydrogen peroxide (−18.8 %) and proline (−29.4 %), lipid peroxidation (−15.9 %), and the activities of superoxide dismutase (−18.2 %), catalase (−21.2 %), and ascorbate peroxidase (−19.1 %). Taken together, the results indicate that a beneficial bacterial consortium comprising Bradyrhizobium spp. and A. brasilense strains combined with MSM application can alleviate oxidative damage during dry spells. Furthermore, this consortium improved soybean nodulation, plant growth development, and grain yield by up to 12.2 %.

1. Introduction
Adverse environmental factors are increasingly impacting agriculture worldwide (FAO, 2019). To guarantee global food security, mechanisms to mitigate the effects of abiotic stresses caused by climate change have been investigated in several crop species (Fukami et al.,

Abbreviations: BNF, biological nitrogen fixation; ROS, reactive oxygen species; MD, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; PGPB, plant growth-promoting bacteria; MSM, microbial secondary metabolites; LCOs, lipo-chitooligosaccharides; IAA, indole-3-acetic acid; ACC deaminase, 1-aminoacyclopropane-1-carboxylate deaminase; A, net photosynthetic rate; gs, stomatal conductance; Ci, internal CO2 concentration in the substomatal chamber; E, transpiration; FW, fresh weight; PH, plant height; NN, nodule number; NDW, nodule dry weight; RDW, root dry weight; SDW, shoot dry weight; GY, grain yield.

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Soybean (Glycine max (L.) Merrill) is an important crop globally because of its use in protein, oil, and biofuel production (Moretti et al., 2020a). In addition, due to its high capacity for biological nitrogen fixation (BNF), soybean plays a key role in agriculture sustainability (Hungria and Mendes, 2015; Ciampitti and Salvagiotti, 2018; Moretti et al., 2018). Soybean grain yield is affected by different environmental conditions, especially pluvial precipitation and temperature (Battisti and Sentelhas, 2017). Variations in the climate characteristics of tropical regions used for cropping (Alvares et al., 2013; Cunningham, 2020) result in differences in the intensity, frequency, and timing of water supply (Hu and Wiatrak, 2012; Battisti and Sentelhas, 2014). Water deficit is a major limiting factor of soybean yield and can be mitigated at least partially by using cultivars with appropriate physiological traits and crop management (Battisti and Sentelhas, 2017).

Several mechanisms of tolerance and resistance (e.g., expression of defence-related genes and phytohormones production) can be adopted by plants to deal with biotic and abiotic stresses. Among them, the major mechanism is to reduce the accumulation of reactive oxygen species (ROS) in plant tissues (dos Reis et al., 2017; Santos et al., 2017). ROS are byproducts of several metabolic pathways in various organelles, including chloroplasts, mitochondria, and peroxisomes (Gill and Tuteja, 2010). ROS include free radicals resulting from the metabolism of oxygen (O$_2$), such as superoxide radicals (O$^2-$), hydrosol radicals (OH-s), hydrogen peroxide (H$$_2$$O$_2$$) and singlet oxygen (O$_2$). In plants, oxidative stress may be relieved by antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC: 1.11.1.6), and ascorbate peroxidase (APX; EC 1.11.1.11). SOD, which dismutates O$_2$ radicals (O$_2$) in the first excited electronic state to H$_2$O$_2$, is the first line of defense against ROS (Gill and Tuteja, 2010); H$_2$O$_2$ can then be detoxified to H$_2$O by CAT and APX through the conversion of H$_2$O$_2$ to H$_2$O and O$_2$ (Alam and Ghosh, 2018).

Stomatal and osmotic adjustments can occur through several mechanisms and channels. Inorganic ions such as sodium (Na$^{+}$), potassium (K$^{+}$), and chloride (Cl$^{-}$) drive most of the osmotic potential in many plant species, while sugars and amino acids, especially proline, are the main osmoregulators in vascular plants (Alexiaga et al., 2001; Ueda et al., 2001). Oxidative damage caused by excess ROS affects cellular components, resulting in membrane disruption via lipid peroxidation (Santos et al., 2017). However, the accumulation of solutes such as proline can protect cells against increased ROS levels (Juge et al., 2012; Mauad et al., 2016). Increased proline biosynthesis in plant tissues contributes to enzymatic stabilization and/or osmoregulation in response to abiotic stress (e.g., salinity, drought, heavy metals, or extreme temperature) (Torelo and Rice, 1986; Abbasi et al., 2013; Mauad et al., 2016).

Plant growth-promoting bacteria (PGPB) and their metabolites, e.g., lipo-chitoooligosaccharides (LCOs) and lipopolysaccharides (LPS), may play a strategic role in alleviating the deleterious effects of ROS through the production of various phytohormones, such as ethylene (Glick, 2012), cytokinins (Tien et al., 1979), gibberellins (Bottini et al., 1989), abscisic acid (Cohen et al., 2006), salicylic acid (Sahoo et al., 2014), indole-3-acetic acid (IAA), and jasmonic acid (Spaepen and Vanderleyden, 2015), among others (Fukami et al., 2018a), leading to the activation of physiological and biochemical mechanisms that confer plant tolerance to abiotic and biotic stresses (Fukami et al., 2018b, c).

Phytohormones produced by PGPB regulate processes and signaling networks in plant organs and may ultimately lead to increased grain yields (Moretti et al., 2020a, b), including antioxidant metabolism (Fukami et al., 2017), exopolysaccharide production (Sandhya et al., 2010), osmotic adjustment (Cerezini et al., 2014), heavy metal mitigation (Bashan and de-Bashan, 2010), plant pathogen biocontrol (Nicholson, 2002), and induced systemic tolerance (Pozo et al., 2008). However, the effects of beneficial microbes and microbial metabolites on soybean under water stress are poorly understood.

We hypothesized that beneficial microbes (Bradyrhizobium spp. with different combinations of A. brasilense, B. subtilis, and rhizobial metabolites) can alleviate the negative effects of dry spells in the field on soybean carbon and antioxidant metabolism by increasing the intrinsic tolerance of soybean plants to abiotic stresses, thereby also improving grain yield. To test this hypothesis in practice, we established soybean under tropical field conditions during two growing seasons (i.e., without water supplementation and in region prone to dry spells during the crop cycle), aiming to mimic the field-real conditions of soybean cultivation in rainfed areas.

2. Materials and methods

2.1. Site description

Two field experiments under rainfed conditions were carried out in the 2016–2017 and 2017–2018 growing seasons with soybean at the Lageado Experimental Farm of São Paulo State University (UNESP), located in the municipality of Botucatu in the southeastern region of São Paulo State, Brazil (48°26′W, 22°51′S, elevation of 786 m above sea level). According to the Brazilian soil classification system (Santos et al., 2018), the soil is classified as a Latosol, which corresponds to the classifications of Ferralsol (Jahn et al., 2006) and Oxisol (Soil Survey Staff, 2014). According to the Köppen-Geiger climatic classification system (Alvares et al., 2013), the region has a mesothermal climate (Cwa), that is, a humid subtropical climate with dry winters and hot summers. The average rainfall is 1,360 mm year$^{-1}$, and the mean annual air temperature is 20.7 °C (50-year average) (Unicamp, 2020). Climatological data for the study period were collected from meteorological stations located near (approximately 400 m) the experimental location. The characterization of short dry spells (approximately 15 days) in southeastern Brazil during the monsoon season (from October to April) were comprehensively established by Cunningham (2020) based on an analysis of a historical series of 37 austral summer seasons (from 1979 to 2016).

Soil texture (Donagema et al., 2017) and chemical (van Raij et al., 2001) properties at a depth of 0–0.20 m were determined prior to the establishment of the experiment and are presented in Table 1. The quantification of the autochthonous bacterial population capable of

| Physical | Clay | g kg$^{-1}$ | 602 |
| Sand | g kg$^{-1}$ | 117 |
| Bulk density | g cm$^{-3}$ | 1.19 |

| Chemical | pH$_{\text{KCl}}$ | 5.10 |
| Organic matter | g kg$^{-1}$ | 26.2 |
| Phosphorus-available (P$_{\text{min}}$) | mg kg$^{-1}$ | 59.0 |
| Calcium (Ca$_{\text{2+}}$) | mmol kg$^{-1}$ | 25.0 |
| Magnesium (Mg$^{2+}$) | mmol kg$^{-1}$ | 15.0 |
| Potassium (K$_{\text{+}}$) | mmol kg$^{-1}$ | 3.90 |
| Aluminum (Al$^{3+}$) | mmol kg$^{-1}$ | 2.00 |
| Potential acidity (H$_2$) | mmol kg$^{-1}$ | 42.0 |
| Sulfate (SO$_4^{2-}$) | mg kg$^{-1}$ | 4.90 |
| Boron (B$_{\text{aq}}$) | mg kg$^{-1}$ | 0.40 |
| Copper (Cu$_{\text{DTPA-TEA}}$) | mg kg$^{-1}$ | 8.80 |
| Iron (Fe$_{\text{DTPA-TEA}}$) | mg kg$^{-1}$ | 22.0 |
| Manganese (Mn$_{\text{DTPA-TEA}}$) | mg kg$^{-1}$ | 26.2 |
| Zinc (Zn$_{\text{DTPA-TEA}}$) | mg kg$^{-1}$ | 2.10 |
| Base saturation (BS) | % | 51.0 |
| Cation exchange capacity (CEC = pH _7.0) | mmol kg$^{-1}$ | 86.0 |

* DTPA-TEA, diethylenetriaminepentaacetic acid-triethanolamine.
* CFU, colony forming units.
nodule efficiency in soybean in the soil was estimated by the most probable number (MPN) using soybean plants as trapping hosts, according to the methodology described by O’Hara et al. (2016).

Liming to increase the base saturation (BS) of the topsoil (0–0.20 m depth) to 70% was conducted approximately 60 days prior to the beginning of the experiment using dolomitic limestone [CaMg(CO$_3$)$_2$] (280 g kg$^{-1}$ of calcium oxide—CaO, 180 g kg$^{-1}$ of magnesium oxide—MgO, and 81% of calcium carbonate equivalents—%E$_{CaCO3}$) according to the methodology described by Quaggio and van Raij (1997).

2.2. Experimental design and treatments

A randomized complete block (RCB) design involving a 4 (inoculant treatments) × 2 (metabolite applications) × 2 (growing seasons) factorial scheme was used with four replicates.

The inoculation treatments factor was represented by (i) standard inoculation (SI) with Bradyrhizobium japonicum (strain SEMIA 5079) + Bradyrhizobium diaeofficiens (strain SEMIA 5080) via seeds at sowing; (ii) SI + foliar spraying with Bacillus subtilis (Bs) (strain QST 713) at the V3 growth stage (Fehr and Caviness, 1977); (iii) SI + foliar spraying with A. brasilense (Ab) (strains Ab-V5 and Ab-V6) at the V1 growth stage; (iv) SI + foliar spraying with Bs at V3 + foliar spraying with Ab at V4. The metabolite applications factor was represented by the presence or absence of application of microbial secondary metabolites of B. diaeofficiens USDA 110 + Rhizobium tropici CFT 899.

It is worth mentioning that annual inoculation of soybean is a practice regularly adopted by the farmers, even in areas that have been inoculated for several years, such that nowadays about 80% of the area cropped with soybean in the country is annually inoculated. Therefore, standard inoculation was considered as the control treatment in our study. The field experiments were conducted in the growing seasons of 2016–2017 and 2017–2018.

2.3. Microbial strains, metabolites and inoculants

2.3.1. Microbial strains

Bradyrhizobium japonicum strain SEMIA 5079 (= CPAC 15, = CNPSo 07) and B. diaeofficiens strain SEMIA 5080 (= CPAC 7, = CNPSo 06) are used in commercial inoculants in Brazil since 1992 (Hungria et al., 2006; Hungria and Mendes, 2015). The Brazilian strain selection program for natural variants of B. japonicum and B. diaeofficiens adapted to environmental conditions was detailed previously by Hungria and Vargas (2000), and the strains used in this study are representative of the majority of inoculants applied to soybean-cropped areas in the country. Inoculation with these strains is practiced even in areas that have received inoculants for several years, as the annual inoculation allows average grain yield increases of 8%. More information about the strains and their performance is available elsewhere (Hungria et al., 2020, 2006; Hungria and Mendes, 2015).

Azospirillum brasilense strains Ab-V5 (=CNPSo 2083) and Ab-V6 (=CNPSo 2084) were selected, validated under field conditions and launched for the use in microbial inoculants for grasses in Brazil in 2009 (Hungria et al., 2020), and for co-inoculation of soybean in 2013. Nowadays estimates are that 25% of the area cropped to soybean in Brazil is co-inoculated with Bradyrhizobium and these two A. brasilense strains. More information about the A. brasilense strains is available elsewhere (Hungria et al., 2010; Santos et al., 2021).

B. japonicum, B. diaeofficiens and A. brasilense strains are deposited at the “Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja” (WFCC Collection # 1213, WDCM Collection # 1054), in Londrina, State of Parana, Brazil. Bacillus subtilis strain QST 713 was developed by Boyer CropScience (European Union, Reg. (EC) no. 839/2008).

2.3.2. Microbial metabolites

The microbial secondary metabolites (MSM) were extracted obtaining by growing individually Rhizobium tropici strain CIAT 899 (= CNPSo 103, = SEMIA 4077) and B. diaeofficiens strain USDA 110 (= CNPSo 56) in modified-YM medium (Hungria et al., 2016) enriched with inducer of nodule genes for each species, resulting in metabolites enriched in lipo-chitoooligosaccharides (LCOs) (Marks et al., 2013, 2015). Both strains are also are deposited at the “Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja”. After centrifugation, the supernatants of each bacterium were lyophilized, resulting in the MSM product. Prior to soybean sowing, the lyophilized MSM were resuspended in a mixture of acetone and water (20%; w/v) (Marks et al., 2015) and mixed in equal parts of metabolites of R. tropici and B. diaeofficiens.

2.3.3. Inoculants

The standard inoculation (SI) was carried out with liquid inoculants containing B. japonicum strain SEMIA 5079 and B. diaeofficiens strain SEMIA 5080 with a concentration of 7 × 10$^8$ cells mL$^{-1}$ and applied to provide 1.2 × 10$^8$ cells seed$^{-1}$. SI is a common practice in soybean cultivation in tropical regions (Hungria et al., 2006, 2020).

The MSM concentration was adjusted to 1.0 mL L$^{-1}$, which corresponds to approximately 10$^8$ M, and applied at rate of 4 mL kg$^{-1}$ of seeds.

Foliar inoculation (by foliar spraying) of B. subtilis strain QST 713 (1 × 10$^9$ cells mL$^{-1}$) was performed at the V3 soybean phenological stage (Fehr and Caviness, 1977) by applying 3 L of the inoculant in 200 L of water to 1 ha. Foliar spraying of A. brasilense strains Ab-V5 (= CNPSo 2083) and Ab-V6 (= CNPSo 2084) at a concentration of 2 × 10$^8$ cells mL$^{-1}$ was carried out at the V4 soybean phenological stage (Fehr and Caviness, 1977) by applying 300 mL of inoculant in 150 L water to 1 ha.

Foliar spraying with A. brasilense strains and B. subtilis was carried out according to the technical recommendations of the manufacturers and was always performed in the late afternoon (5:00 pm).

2.4. Crop management practices history

Prior to soybean (cultivar TMG 1264 RR; Tropical Breeding & Genetics, Cambé, PR, Brazil) sowing (Semeato, model Personale Drill 13, Passo Fundo, RS, Brazil) the experimental area was cultivated with black oats (Avena strigosa Schreb.) to provide straw for the no-tillage system. Each plot consisted of 10 rows spaced 0.45 m apart, leading to a total plot area of 45 m$^2$. The plots were separated by 0.5-m-wide rows and 1.5-m-wide terraces to avoid cross-contamination (bacteria and fertilizer) from rainfall surface runoff. The soybean plants were fertilized with 300 kg ha$^{-1}$ of 00–20–20 (60 kg ha$^{-1}$ of P$_2$O$_5$ and 60 kg ha$^{-1}$ of K$_2$O) in both growing seasons. Seeds were treated with fungicides (carboxin + thiram at 100 g + 100 g a.i. 100 kg$^{-1}$ seeds) prior to inoculation and sowing. Seed inoculation was performed 1 h before sowing. Foliar inoculations were carried out with a tractor-mounted sprayer. At phenological stage V$_4$ (Fehr and Caviness, 1977), all treatments received a foliar application of 20 g ha$^{-1}$ of molybdenum (Mo) (as Na$_2$MoO$_4$.2H$_2$O) and 2 g ha$^{-1}$ of cobalt (Co) (as CoCl$_2$.6H$_2$O), which are required to maximize BNF with soybean (Embrapa, 2020). The management of weeds, insects, and diseases were carried according to the recommendations of Embrapa (2020) when necessary so these were not limiting factors.

2.5. Sampling, measurements and data analyses

2.5.1. Soil water content

Prior to the establishment of the experiment, the soil water-holding capacity was determined according to the tension table and the Richards extractant chamber methods (Casel and Nielsen, 1986), which allowed the determination of the soil water potential ($\theta_{wp}$). The climatological water balance was monitored and calculated via electronic spreadsheets according to the methods proposed by Rolim et al. (1998) following the
procedure of Thornthwaite and Mather (1995) to determine the real evapotranspiration (ETr). The climatological water balance of the two experimental growing seasons is shown in Fig. 1.

In both growing seasons, at the R_5 growth stage (beginning of seed development) (Fehr and Caviness, 1977), which corresponds to seeds with a length of approximately 3.0 mm in the pod at one of the four uppermost nodes on the main stem, photosynthetic pigments, gas exchange, oxidative stress, antioxidant metabolism, and proline content were measured in the third fully expanded leaves of plants under moderate drought from short dry spells.

2.5.2. Photosynthetic pigments

Chlorophyll a (chl a), chlorophyll b (chl b), total chlorophyll (total chl) and total carotenoid (total car) contents were determined in the third fully expanded leaves from five discs cut between the edge and the central rib of the soybean leaves using a paper punch (0.5 cm in diameter) at the R_5 growth stage. The discs were incubated for 24 h in capped glass vials containing 2 mL of N, N-dimethylformamide (DMF) and wrapped in aluminum foil according to the methodology proposed by Lichtenthaler (1987). Pigment concentrations were quantified using the spectrophotometric method at wavelengths of 664, 647, and 480 nm for chlorophyll a, b and carotenoids, respectively. The pigment concentrations were determined as proposed by Wellburn (1994).

2.5.3. Gas exchange

Gas exchange was evaluated via nondestructive analysis with a portable gas exchange device (LI-6400 infrared gas analyzer (IRGA), LI-COR Biosciences Inc., Lincoln, NE, USA). Samples were taken from the central leaflet of the third fully expanded leaves and intact trifoliate leaf from the plant apex of the main stem of 10 plants per plot. The parameters of the instrument were as follows: 380–400 mol mol⁻¹ atmospheric CO₂, 1100 μmol m⁻² s⁻¹ of photosynthetically active radiation (PAR) supplied by LED lamps, 25–27 °C leaf chamber temperature, and 60–70 % relative humidity. The minimum equilibration time for each set of measurements was 3 min. The measurements were performed between 10:00 and 12:00 am. The following parameters were determined: net photosynthesis rate (A; μmol CO₂ m⁻² s⁻¹), stomatal conductance (gs; mol H₂O m⁻² s⁻¹), internal CO₂ concentration in the substomatal cavity (Ci; μmol mol⁻¹), and transpiration (E; mmol H₂O m⁻² s⁻¹).

2.5.4. Oxidative stress and antioxidant metabolism

To evaluate the contents of H₂O₂ and malondialdehyde (MDA) as well as the activities of SOD (EC:1.15.1.1), CAT (EC:1.11.1.6) and APX (EC:1.11.1.11), sampling was performed on the same leaflets taken at the R_5 growth stage to assess the gas exchange parameters. The sampled tissue was then flash-frozen in liquid N and stored at −80 °C.

Lipid peroxidation was evaluated according to the method of Heath and Packer (1968). To calculate MDA content, a molar extraction coefficient of 155 mM⁻¹ cm⁻¹ was used, and the results were expressed in nanomoles of MDA per gram of fresh weight (FW). The H₂O₂ content was determined according to the method of Alexieva et al. (2001). The content was calculated based on a calibration curve and expressed in micromoles of H₂O₂ per gram of FW. Extractions for enzyme analysis were performed according to the methodology described by Silva et al. (2020). SOD activity was evaluated as described by Giannopolitis and Ries (1977), and the results were expressed in units (U) of SOD per milligram of protein. CAT activity was evaluated as described by Azavedo et al. (1998), and the results were expressed in micromoles per minute per milligram of protein. APX activity was evaluated according to Gratão et al. (2008), and the results were expressed in nanomoles per minute per milligram of protein.

2.5.5. Proline content

Proline content was determined according to Torello and Rice (1986) in the same leaflets collected at the R_5 growth stage used for the other analyses. The absorbance at wavelengths of 647 and 664 nm was determined via a spectrophotometer, and the results were expressed per gram of FW, as described by Mauad et al. (2016).

2.5.6. Crop yield

Additionally, at the R_5 stage, five plants per plot were harvested and separated into shoots and roots according to the methodology described by Hungria et al. (2006). In the laboratory, shoots were separated from roots and the latter were carefully washed and placed in a forced-air dryer at 65 °C until constant weight was obtained (approximately 72 h). Nodules were removed from roots and dried again. From this material, the nodule number (NN), nodule dry weight (NDW), root dry weight (RDW), and shoot dry weight (SDW) were determined.

At physiological maturity, plants were harvested from a 15-m² area from the central part of each plot and estimated the final population of plants, plant height, position of insertion of the first pod, numbers of branches and pods per plant, and number of grains per pod. Grain yield (kg ha⁻¹) and thousand-grain weight (W1000) were converted to values on a dry weight basis by correcting for 13 % moisture. The moisture was determined with an automatic measuring device (Gehaka G650i, Brazil).

2.6. Statistical analyses

All data were initially analyzed via the Shapiro-Wilk test (Shapiro and Wilk, 1965) for normality and the Levene test for homoscedasticity (Levene, 1960), both at p < 0.05; the UNIVARIATE procedure of SAS version 9.4 was used for the analysis (SAS Institute, 2015). The data were also tested for sphericity by the Bartlett test (Bartlett, 1937) via the FACTOR procedure of SAS version 9.4 (SAS Institute, 2015). The results indicated that all data were distributed normally (W ≥ 0.90) and exhibited no sphericity. The data were then analyzed with the linear
mixed-effect model by the PROC MIXED procedure of SAS and with the Satterthwaite approximation to determine the denominator degrees of freedom for the fixed-effects tests. The blocks and spatial interactions between neighbouring blocks were considered as random effects, whereas bacterial consortium, microbial metabolites, growing season, and their interactions were considered as fixed effects. The results were reported as the least square means and were separated by the probability of differences option (PDIFF). The means were compared using the least significant difference (LSD) test. The main factors and interactive effects were considered statistically significant at \( p \leq 0.05 \). The heatmap was built calculating the Pearson correlation coefficients (\( p \leq 0.05 \)) and only the significant correlations are shown.

3. Results

3.1. Photosynthetic pigments

The photosynthetic pigments of soybean plants did not differ between those that received SI plus inoculation with \( B.\ subtilis \) and those that received SI (\( Bradyrhizobium \) spp. only) (Table S1). However, plants inoculated with \( A.\ brasilense \) strains exhibited higher concentrations of these pigments compared to plants that were not inoculated with \( A.\ brasilense \), and application of MSM further increased these levels. A significant interaction between bacterial consortium and MSM application was observed (\( p \leq 0.01; \) Table S1) for both chlorophyll and carotenoid contents in soybean leaves at the R5 reproductive stage in both growing seasons (Fig. 2). Plants treated with SI plus \( A.\ brasilense \) and MSM exhibited increases in chla, chlb, total chl, and total car contents of up to 14.5 %, 30.8 %, 17.2 %, and 27.3 %, respectively, compared with the plants receiving SI only. The results for the two growing seasons were similar.

3.2. Gas exchange

The effects of the different treatments on gas exchange parameters were similar to those on photosynthetic pigments, highlighting the benefits of foliar spraying of \( A.\ brasilense \) and MSM application (Table S2). Interaction effects of bacterial consortium and MSM inoculation on the net photosynthesis rate \( (A) \) (\( p \leq 0.01 \)), stomatal conductance \( (g_s) \) (\( p \leq 0.01 \)), internal CO\(_2\) concentration in the substomatal cavity \( (CI) \) (\( p \leq 0.01 \)) and transpiration \( (E) \) (\( p \leq 0.01 \)) were detected (Table S2). Additionally, \( A.\ brasilense \), \( g_s \), \( CI \), and \( E \) increased significantly by up to 17.7 %, 56.5 %, 8.3 %, and 44.0 %, respectively, in all plants receiving SI plus foliar spraying of \( A.\ brasilense \) strains and MSM application (Fig. 3A-D) compared to plants receiving SI alone.

3.3. Oxidative stress, antioxidant metabolism and proline content

Interaction effects of bacterial consortium and MSM inoculation on \( \text{H}_2\text{O}_2 \) content (\( p \leq 0.01 \)), lipid peroxidation (assayed via the MDA content) (\( p \leq 0.01 \)), SOD (\( p \leq 0.01 \)), CAT (\( p \leq 0.01 \)), and APX (\( p \leq 0.01 \)) activities, and proline content (\( p \leq 0.01 \)) in soybean leaf tissue were detected (Table S3). In addition, \( \text{H}_2\text{O}_2 \) content, lipid peroxidation rates, and proline content were highest in plants receiving SI only (Fig. 4A-B). By contrast, plants receiving SI plus foliar spraying with \( A.\ brasilense \) strains and MSM application exhibited 18.8 % lower \( \text{H}_2\text{O}_2 \) content, 29.4 % lower proline content, and 15.9 % lower lipid peroxidation in leaf cell membranes. Moreover, this treatment resulted in significant decreases in SOD (\( \sim -18.2 \% \)), CAT (\( \sim -21.2 \% \)), and APX (\( \sim -19.1 \% \)) activities compared to SI treatment alone (Fig. 4C-E).

![Fig. 2](image-url) Chlorophyll a (chl a), chlorophyll b (chl b), total chlorophyll (total chl), and total carotenoids (total car) in soybean leaves at the R5 reproductive stage as a function of bacterial consortium and microbial metabolites under field conditions. Different lowercase letters indicate significant differences between treatments, and different uppercase letters indicate significant differences between the presence and absence of application of microbial secondary metabolites by Fisher’s least significant (LSD) test at \( p \leq 0.05 \). The error bars express the standard error of the mean of the two growing seasons (\( n = 8 \)).
3.4. Crop yield

Positive interactions effects of bacterial consortium (Bradyrhizobium plus A. brasilense strains) and rhizobial metabolites application were observed on soybean nodule number (NN, 19 %), nodule dry weight (NDW, 21 %), root dry weight (RDW, 17.2 %) and shoot dry weight (SDW, 20.6 %) when compared to exclusively SI (p ≤ 0.01, Table S4) (Fig. 5 A-D). At physiological maturity, significant interactions effects of bacterial consortium and MSM application on soybean plant height (PH), number of pods, thousand-grain weight (W1000) and grain yield (GY) were detected (p ≤ 0.01, Table S5). Plant height, pods, W1000 and GY increased considerably when plants received SI plus foliar application of A. brasilense strains and MSM application, by up to 7.6 % (PH), 25.3 % (pods), 5.0 % (W1000) and 12.2 % (GY), respectively, compared to SI alone (Fig. 6 A-D). No benefits of foliar application of B. subtilis were observed. Overall, significant effects were not observed in the final population of plants (mean = 288,886 plant ha⁻¹), position of insertion of the first pod (mean = 12 cm), number of branches per plant (mean = 3), nor in the number of grains per pod (mean = 2.2) (data not shown).

3.5. Correlation analysis between plant measurements

Changes in pigments concentrations correlated positively with all photosynthetic parameters from soybean gas exchange measurements, and both correlated with soybean plant responses, including root nodulation and dry weight, as well as production of shoot, pods and grains (Fig. 7). On the other hand, oxidative stress and antioxidant metabolism correlated negatively with all other parameters studied and positively with themselves.

4. Discussion

For successfully realizing attainable yield, soybean cultivation in South America requires 450–800 mm of rainfall (Embrapa, 2020). The maximum crop requirement for water occurs between flowering and grain filling, with ETr of ~7–8 mm day⁻¹ (Embrapa, 2020) and a crop coefficient (Kc) of ~1.5 (Farias et al., 2001). In the present study, total rainfall was 789 and 705 mm in the 2016–2017 and 2017–2018 growing seasons, respectively. However, there was an uneven distribution of rain during the crop cycle. Short dry spells with negative hydric balance lasting 17 and 14 days were observed in the first and second growing seasons, respectively, both prior to the R5.1 reproductive stage (Fig. 2) and during the critical stage of early grain filling. According to Flexas et al. (2004), the stress level can be quantified via stomatal conductance, where values ≥ 0.2 mol H₂O m⁻² s⁻¹ represent well-watered plants, values of 0.1–0.2 mol H₂O m⁻² s⁻¹ represent moderate drought-stressed plants, and values ≤ 0.1 mol H₂O m⁻² s⁻¹ represent severely drought-stressed plants. According to this classification, in both growing seasons the plants treated with SI only were under moderate drought stress at the R5 stage.

Short dry periods during the summer cropping season are common in tropical regions (Silva et al., 2019) and, combined with the high temperature and evapotranspiration typical of this period of the year, greatly impact crop productivity, particularly during critical periods of plant development such as flowering and grain filling (Basu et al., 2016). In addition, water restriction during the reproductive stage (R₁–R₅; beginning of flowering until grain formation) leads to several physiological, morphological, and biochemical changes resulting in loss of leaf turgor, premature flower dropping, pod abortion and ultimately lower grain yields (Nelson et al., 2005).

Chlorophyll is the main photosynthetic pigment in the
Calvin–Benson–Bassham (CBB) cycle (Croft et al., 2017; Busch, 2020). Reduced chlorophyll content decreases the light energy uptake and light utilization capacity of plants, resulting in a decrease in photosynthesis (Ansari et al., 2019). Carotenoids are responsible for plastid adaptation to stress conditions such as light and energy dissipation and avoiding excessive ROS production (Rodriguez-Concepcion et al., 2018). One of the main effects of drought in higher plants is reduction and/or inhibition of photosynthesis (Reddy et al., 2004), as reflected by decreased synthesis of chlorophyll and carotenoids. As schematized in Fig. 8, our field experiments in this tropical region clearly demonstrated that the bacterial consortium represented by seed treatment with *Bradyrhizobium* spp. and rhizobial metabolites (MSM) and foliar application of *A. brasilense* at the V₄ stage increased photosynthetic pigments, namely, chl a, chl b, total chl, and total car. However, the increased growth of plants inoculated with MSM could be at least partially related to indirect effects of LCOs on photosynthesis and plant growth acceleration by stimulating mitotic activity in the meristematic tissue of leaves (Khan et al., 2020).

Our results also demonstrated that, compared with plants inoculated exclusively with *Bradyrhizobium* spp., the combination of *Bradyrhizobium* spp. inoculation with *A. brasilense* and MSM treatment improved the efficiency of gas exchange, including stomatal conductance and transpiration. Regulating stomatal conductance under conditions of low hydric availability is key to improving water-use efficiency (Gupta et al., 2020). Our results suggest that *A. brasilense* plus MSM positively influenced stomatal opening and closing, which directly affected the process of gas exchange and contributed to increased biomass accumulation. A previous study conducted on the same site also reported important roles of MSM in *Bradyrhizobium*-soybean symbiosis (Moretti et al., 2020b). This previous study suggested that a more developed root system

![Graphs showing data on photosynthetic pigments](image-url)
In the present study, the H_2O_2 oxidant metabolism are activated to inactivate ROS (Farooq et al., 2019). ROS can severely damage the photosynthetic apparatus, resulting in lipid peroxidation (Loix et al., 2018). To mitigate the effects of abiotic stresses, intracellular ROS removal, reduced membrane peroxidation and activities of antioxidant enzymes (CAT, APOX, POD, and SOD) increased in response to drought stress but significantly lower in soybean leaves of plants treated with the consortium on avoiding oxidative damage and reducing the accumulation of proline in tissues under water stress. Similar results were reported in wheat (Triticum aestivum L.) with a consortium of seeds inoculated with Azospirillum spp. with the plant growth regulators salicylic acid and putrescine, indicating physiological adjustments to reduce drought impacts, and our results support this assumption. Fukami et al. (2017) reported that proline content, lipid peroxidation and activities of antioxidant enzymes (CAT, APOX, POD, and SOD) increased in response to drought stress but decreased due to a consortium of Bacillus spp. with the plant growth regulators salicylic acid and putrescine, indicating physiological adjustments to reduce drought impacts, and our results support this assumption. Fukami et al. (2018b) reported that inoculation of maize with Azospirillum reduced proline synthesis, and the decrease in proline content was correlated with the maintenance of plant metabolism under abiotic stress. Similar results were reported in wheat (Triticum aestivum L.) with a consortium of seeds inoculated with Planomicrobium chinense and Bacillus cereus followed by foliar spray of salicylic acid one month later, where leaf proline content, lipid peroxidation and antioxidant enzyme activity were decreased, and drought effects were decreased (Khan and Bano, 2019).

Drought stress significantly affected the concentration of chlorophyll and carotenoids (Fig. 2), photosynthetic capacity and carbon assimilation of plants (Fig. 3), influencing root and shoot the dry weight (Fig. 5), seriously reflecting 1000-grain weight (Fig. 6). The photosynthates produced in the source leaves, mainly sucrose, are the main components of C metabolism and amino acid biosynthesis for the development of the grains (Du et al., 2020). Water stress during the grains filling phase inhibited the synthesis and accumulation of carbohydrates, probably by blocking cell division (Awasthi et al., 2014; Sehgal et al., 2017), which increases soil exploration and, in turn, the distribution and activity of soybean roots, which might indirectly ensure plant metabolic activities and soybean establishment and favor tolerance to moderate water restriction.

The gradual loss of photosynthetic capability due to drought conditions results in accumulation of electrons in photosystems I and II, leading to increased ROS levels and lipid peroxidation (Croft et al., 2017). ROS can severely damage the photosynthetic apparatus, resulting in lipid peroxidation (Loix et al., 2019). In the present study, the H_2O_2 concentration and lipid peroxidation rate in soybean leaves were greater in plants inoculated just with Bradyrhizobium spp. and with B. subtilis by foliar spraying at the V_3 stage. By contrast, the lower activities of SOD, CAT and APX in soybean leaves of plants treated with Bradyrhizobium spp. + MSM + A. brasilense indicate an increased ability to mitigate ROS compared with inoculation with Bradyrhizobium alone. Indeed, SOD, CAT and APX activities have been used as important indicators of PGPR-induced plant resistance to abiotic stresses, intracellular ROS removal, reduced membrane peroxidation, stabilization of membrane permeability and improved plant photosynthesis (Sandhya et al., 2010; Fukami et al., 2018b, c). Interestingly, in chickpea (Cicer arietinum L.) Khan et al. (2019) reported that proline content, lipid peroxidation and activities of antioxidant enzymes (CAT, APOX, POD, and SOD) increased in response to oxidative stress genes in leaves (APX1, APX2, SOD2, SOD4); however, the up-regulation of these genes was always greatest when foliar spraying was combined with the application of rhizobial metabolites. This previous study corroborates our results and suggests that the persistence of oxidative stress is reduced when live cells (only Azospirillum spp.) are applied in combination with rhizobial metabolites.

Proline is an important stress marker molecule associated with osmoregulation that prevents dehydration under negative soil water potential (ψ_ω) (Goswami et al., 2016). In our study, proline content was significantly lower in soybean leaves of plants treated with the consortium comprising Bradyrhizobium spp., MSM and A. brasilense strains compared with plants treated with SI alone, suggesting an important impact of the former consortium on avoiding oxidative damage and reducing the accumulation of proline in tissues under water stress. Similar, Fukami et al. (2018b) reported that inoculation of maize with Azospirillum reduced proline synthesis, and the decrease in proline content was correlated with the maintenance of plant metabolism under abiotic stress. Similar results were reported in wheat (Triticum aestivum L.) with a consortium of seeds inoculated with Planomicrobium chinense and Bacillus cereus followed by foliar spray of salicylic acid one month later, where leaf proline content, lipid peroxidation and antioxidant enzyme activity were decreased, and drought effects were decreased (Khan and Bano, 2019).
Fig. 6. Plant height, pods, soybean 1000-grain weight (W1000) and grain yield (GY) as a function of bacterial consortium and microbial metabolites under field conditions. Different lowercase letters indicate significant differences between treatments, and different uppercase letters indicate significant differences between the presence and absence of application of microbial secondary metabolites by Fisher’s least significant (LSD) test at $p \leq 0.05$. The error bars express the standard error of the mean of the two growing seasons ($n = 8$).

Fig. 7. Heatmap of the Pearson correlation coefficients matrix between soybean physiological, biochemical and agronomic parameters. Only significant correlations at $p \leq 0.05$ are shown. Chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Total chl), total carotenoids (Total car), net photosynthesis rate (A), stomatal conductance (gs), internal CO$_2$ concentration (Ci), transpiration (E), hydrogen peroxide (H$_2$O$_2$), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), proline, nodule number (NN), nodule dry weight (NDW), root dry weight (RDW), shoot dry weight (SDW), plant height (PH), pods, thousand-grain weight (W1000) and grain yield (GY).
reduced energy demand and promoted the early completion of the grain filling process, resulting in considerable losses in productivity (Fig. 6). In this study, plants treated only with SI presented significant decrease in nodulation, inhibition of root and shoot growth development, photosynthetic activity, and decrease in thousand-grain weight and yield. Taken together (Fig. 7), the results indicate that a beneficial bacterial consortium comprising *Bradyrhizobium* spp. and *A. brasilense* strains combined with MSM application help plants in the accumulation of osmolytes, antioxidants metabolism and upregulate stress-responsive gene for a better acquisition of drought tolerance, ensuring nodulation, root and shoot development, improving the photosynthetic activity of the plant and the production of photoassimilates and, finally, the filling of the grains.

Benefits in plant growth, yield and tolerance to abiotic and biotic stresses with the use of consortia of plant growth-promoting bacteria and microbial metabolites or other synthetic plant-growth regulators have been increasingly reported (e.g., Fukami et al., 2017; Khan et al., 2019; Khan and Bano, 2019; Marks et al., 2015, 2013; Moretti et al., 2020b, 2020a). Recently, Liu et al. (2020) proposed an interesting approach based on observations of microbiomes of plants submitted to stresses, the concept of a "DefenseBiome", that would include the design, construction and application of beneficial microbial synthetic communities; however, as shown in the studies mentioned above as well as in ours, this could also be reached by searching the right combinations of microbial cells and metabolites.

In our study, the beneficial effects of the consortium of *Bradyrhizobium* spp., MSM and *A. brasilense* strains on alleviating soybean water stress under field conditions resulted in improvements in the 1000-grain weight and in grain yield by up to 574 kg ha⁻¹ compared with inoculation with *Bradyrhizobium* spp. alone. Most importantly, as in other studies using different consortia of bacteria with microbial metabolites or synthetic plant regulators (Fukami et al., 2017; Khan et al., 2019; Khan and Bano, 2019), abiotic stresses may be mitigated. Therefore, the consortium identified in our study represents an effective strategy to increase soybean grain yield by improving plant osmotic adjustment, maintenance of physiological processes and biological nitrogen fixation during the short dry spells that occur frequently in tropical regions. This study illustrates the feasibility of identifying beneficial bacterial consortia that can promote plant growth and elicit plant tolerance to abiotic stress, which represents an important biotechnological strategy to achieve sustainable food production and security.

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**CRedit authorship contribution statement**

Luiz Gustavo Moretti: Conceptualization, Methodology, Data curation, Software, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. Carlos Alexandre Costa Crusciol: Supervision, Project administration, Funding acquisition. João William Bossolani: Visualization, Data curation, Writing - review & editing. Juliano Carlos Calonego: Methodology, Data curation, Software. Adonis Moreira: Methodology, Data curation, Software. Ariani Garcia: Visualization, Data curation, Writing - review & editing. Letusa Momesso: Visualization, Data curation, Writing - review & editing. Eiko Eurya Kuramae: Supervision, Project administration, Funding acquisition. Mariangela Hungria: Supervision, Project administration, Funding acquisition.
Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

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