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Vineyard endogenous Myroides odoratimimus and Arthrobacter nicotinovorans have Plant Growth Promoting potential

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Abstract

Climate change is a pivotal challenge to Mediterranean viticulture, including that of the Douro Demarcated Region in Portugal. Identifying and using native beneficial microorganisms emerges as a sustainable, innovative strategy to help vineyards cope with increased temperature and/or drought. From a collection of ~ 400 native bacteria isolated from the Douro vineyards, the screening of siderophore production, phosphate solubilisation, IAA production, and ACCd activity showed that *Myroides odoratimimus* and *Arthrobacter nicotinovorans* were the most promising to act as plant growth promoters (PGP), distinguishing themselves by producing high levels of IAA, and by high phosphate-solubilising capacity, respectively. These strains were applied to the rhizosphere of grapevine plants, and showed positive effects by preventing or increasing the grapevines' reactiveness to the impact of drought stress, on the oxidative disturbance, and the maintenance of the photosynthetic capacity, which permitted the maintenance of energy storage through TIS accumulation, contrary to the results observed in PGP-untreated grapevines subjected to drought stress. Our results demonstrate that enriching grapevines with native *M. odoratimimus* and *A. nicotinovorans* contributed, by different mechanisms of action, and with different impacts, to the plant's defence against drought. These results could be promising towards the development of new strategies, namely the application of these PGP bacteria, to mitigate the impact of climate change on viticulture.

1. Introduction

Climate change is challenging current viticultural practices and products (Keller, 2023; van Leeuwen & Darriet, 2016). Among effects associated with climate change are the increase in average temperature and extreme events, as well as the decrease in precipitation, which cannot be overlooked in grapevine cultivars in the Mediterranean region. Thus, new, more efficient, precise, and sustainable techniques must be employed to ensure the vitality of viticulture. The Douro Demarcated Region (DDR) is acknowledged as one of the most important viticultural regions in the Mediterranean basin, producing distinctive wines, including the Protected Designation of Origin Port, Red, and White wines (IVDP, 2024). However, winemaking in the DDR could be under threat, as it is considered a climate change hotspot, with current projections indicating rising temperatures, decreased precipitation levels, and an increased frequency of extreme climatic events in the coming decades (del Pozo et al., 2019). As a result, serious consequences are expected in the grapevine's vegetative cycle, mainly a production and yield shortage, accompanied by earlier harvesting periods (Santos et al., 2020). Increased temperatures bring a significant impact to winemaking, as they accelerate the vegetative cycle, including maturation, causing accelerated loss of acidity (mainly due to the use of malic acid as an energy source), higher sugar accumulation, and lower accumulation of phenolic compounds, mainly due to their accelerated degradation but also because of the shorter timeframe for their accumulation (Arias et al., 2022; Rogiers et al., 2022). The lower water availability will lead to increased drought stress, lower photosynthetic performance, smaller plants with fewer clusters, and ultimately, smaller grapes with poor solute content (Bertolino et al., 2019; Chaves et al., 2002; Gambetta, 2016; Shehata, 2024). Ultimately, it will cause the

loss of fresh aromas and produce wines with low organoleptic richness, with higher sugar and alcohol contents, distorting the products' traditional characteristics, linked to a singular *terroir*.

Several adaptation strategies have already been developed, including short-term and long-term strategies, which involve cultural/structural practices, as well as water and soil management. More innovative strategies include targeted application of beneficial microorganisms (Prada et al., 2024). Regarding the latter, it is understood that many bacteria have a role in manipulating soil characteristics, mainly by participating in several nutrient cycles, which directly or indirectly influence the grapevines established in that soil. Expectedly, correlations can be established between specific microbiomes and the regional characteristics of the produced wine (Griggs et al., 2021). In this case, some microorganisms facilitate the grapevine's uptake of nutrients and water, characterised as Plant-Growth Promoters (PGP). Some of the most common bacterial genera found to be enhancing grapevine performance, as biofertilisers or biostimulators, include *Azotobacter*, *Rhizobium, Azospirillum*, *Streptomyces, Bacillus*, *Pseudomonas*, and *Pantoea*. Some of them are free-living, such as *Azotobacter* or *Azospirillum*, whilst others have symbiotic relationships with other plants, such as *Rhizobium*, which is mandatory for its capacity to fix N_2, as nitrogenases can only perform in the micro-environment found inside plant roots. These bacteria act as PGP, mainly by enhancing N_2 fixation, PO_4 solubilisation (Vinale et al., 2008), siderophore production, auxin production, S oxidation, K solubilisation, or by limiting ethylene production by the synthesis of 1-aminocyclopropane-1-carboxylate deaminase (ACCd) (Ferreira et al., 2019). Furthermore, Acidobacteria are essential in the carbon cycle, as they can break cellulose and lignin, while *Rhizobium* and *Bacillus* have important roles as plant-associated bacteria, due to their capacity to synthesise hormones [indoleacetic acid (IAA), gibberellic acid, ethylene, and cytokinin, promoting cellular division, differentiation, and shoot development], and increase chlorophyll and carotenoids synthesis (Rahman et al., 2021). These bacteria, by facilitating nutrient uptake, promote grapevine nutrition and, consequently, enhance their performance and resilience, as well as their products' quality, ultimately being an important part of the local *terroir*. However, the ability of these bacteria to perform is also dependent on their origin, as autochthonous bacteria are reported to have higher results, due to their adaptation to the environmental conditions of a specific region, being more adapted to the competition for resources, to soil characteristics, and to the grapevine's specific exudate composition (Backer et al., 2018; Chauhan et al., 2023).

The main goal of this work is to assess and identify promising autochthonous bacteria, regarding their plant-growth-promoting potential, in the microbiome of the rhizosphere of vineyards in the Douro Demarcated Region. Whilst several bacteria are well documented for their PGP capacities, the potential of non-model, region-specific rare taxa from extreme *terroirs*, such as those in the DDR, remains underexplored. This exploration is of the highest value as, being well-adapted to the DDR climatic conditions, the PGP bacteria found here could be a sustainable, ecological approach to help grapevines cope with the future climatic challenges in this wine region, which are mostly related to drought stress caused by decreased precipitation volumes.

2. Materials and Methods

2.1 Sampling Sites and Procedure

The soil sampling for the bacterial collection on the grapevine's rhizosphere was performed in four vineyards in different sub regions in the DDR, encompassing Baixo Corgo, Cima Corgo, and Douro Superior. The vineyards were Quinta do Cavernelho (CV) in Baixo Corgo sub region (41°17'38.62" N, 7°43'13.06" W), Quinta do Seixo (SX) in Cima Corgo sub region (41°09'59.37" N, 7°33'9.82" W), and Quinta Belém (BL, 41°06'12.0" N, 7°07'27.1" W) and Quinta da Ervamoira (EM, 41°01'23.3" N, 7°06'51.8" W), in Douro Superior sub region. Figure 1 provides a clear representation of the location of each sampled vineyard. Samples were collected at around 20–30 cm deep and 10 cm away from the main trunk of each grapevine. A total of six samples were collected per vineyard, each in a different grapevine rhizosphere, so that the broadest possible collection could be obtained. The samples were collected into sterile sampling bags and transported on ice until further use. Afterwards, in 15 mL Falcon tubes, 5 g of each sample was added to 10 mL of sterile water, which served as a stock solution. The stock solution was then diluted (1:10) three times, obtaining four solutions with different soil sample concentrations (stock and 1:10, 1:100, and 1:1000 dilutions from the stock). Finally, 10µl of each soil sample solution was cultured in Petri plates composed of agarised Luria-Bertani (LB) Broth medium with 0.05% cycloheximide (Panreac), to promote bacterial growth and avoid fungal contamination. After three days, phenotypically different bacterial colonies were isolated into new LB agar plates for pure culture development. Once pure cultures were obtained, bacteria were added to liquid LB media for 2 days (~ 20℃), and then 700 µl of each liquid culture was added to 300µl glycerol in cryopreservation tubes. A total of 411 isolates were obtained and identified with the "B" codes, from 001 to 411. The collection was maintained at -80℃ until further use.

2.2 Qualitative screening for Siderophores and Phosphate solubilisation phosphate

Acknowledging the high number of obtained isolates, a preliminary screening was deemed necessary. Two qualitative protocols were used to understand if the isolates could produce siderophores and/or solubilise phosphate. Siderophore production was assessed according to Rehan et al. (2022), with the following modifications. Briefly, 60.5 mg of Chrome azurol S (CAS) was dissolved in 50 mL of dH_2O and then mixed with 10 mL of iron (III) solution (1 mmol/L $FeCl_3.6H_2O$, 10 mmol/L HCl). Also, 72.9 mg of Hexadecyltrimethylammonium bromide (HDTMA) was dissolved in 40 mL of dH_2O and slowly mixed, under stirring conditions, with the first solution, obtaining the CAS reagent. Afterwards, the CAS reagent was added to freshly autoclaved King's Broth (KB) agar (cooled to 60℃), adding 1 part CAS to 9 parts KB agar. The media was then poured into 24-well plate wells, and plates were left to dry in sterile conditions and then stored at 4℃ until further use. Each isolated strain was inoculated into 5 mL of LB broth overnight (~ 25℃), at 180 rpm. Strains were then inoculated in 24-well plates by pipetting 5 µl of each bacterial suspension into each well. Plates were incubated at 28℃ for 3 days, and results were

registered at the end, where a change in the colour of the media, from blue to yellow, was considered a positive result for siderophore production.

Regarding phosphate solubilisation, the qualitative assessment was performed according to Paul & Sinha (2017). In this case, Pikovskaya's agar media was prepared and then poured into 24-well plates, which were dried under sterile conditions, and stored at 4°C until further use. Then, each isolated strain was inoculated into 5 mL of LB broth overnight (~ 25°C), at 180 rpm. Strains were then inoculated in 24-well plates by pipetting 5 µl of each bacterial suspension to each well. Plates were incubated at 28°C for 7 days, and results were registered, where a clear halo surrounding the colonies was considered a positive result for inorganic phosphate solubilisation.

Only the bacteria that showed positive results in both protocols (qualitative siderophore production and phosphate solubilisation) were selected for the further biochemical traits.

2.3 Quantitative screening of siderophore production, phosphate solubilisation, IAA synthesis, and qualitative ACCd activity, and potential PGP bacteria selection.

After the first screening, the nine bacterial strains were assessed for PGP potential with four different protocols: quantitative siderophore production, phosphate solubilisation, IAA synthesis, and 1-aminocyclopropane-1-carboxylate deaminase (ACCd) activity. Regarding siderophore production, the method described by Ambrosini & Passaglia (2017) was used. Briefly, bacterial strains were grown overnight in King B broth, and OD600 was calibrated to 0.1. Then, 10 µl of each strain was inoculated in KB + CAS reagent Petri plates for 48 h at 28°C. Finally, bacterial cultures that produce siderophores formed a yellow halo around the colony, which was measured. The halo diameter is correlated to the capacity of siderophore production. Phosphate solubilisation was quantified according to Kavamura et al. (2013), with some modifications. Bacterial solutions were grown overnight in LB broth at 28°C, under stirring conditions (150 rpm), and then centrifuged at maximum speed for 10 minutes. Precipitated bacteria were washed in 3 mL of Tris-HCl 0.1 M, pH 7.6. After washing, bacteria were suspended in 1 mL of the same buffer, and OD600 was calibrated to 0.1. Then, 100 µL of each strain was inoculated in 10 mL of Pikovskaya's media, for 8 days at 180 rpm and 28°C. Afterwards, 750 µL of each sample was transferred to 1.5 mL tubes with 250 µL of dH2O, vortexed, and centrifuged at 10,000 rpm for 5 minutes. Ultimately, phosphate solubilisation was quantified by spectrophotometry by adding, in a 96-well plate, 20 µl of the supernatant of each strain to 20 µl of ammonium molybdate + malachite green solution, 10 µl of polyvinyl alcohol, and 120 µl of dH_2O (three technical replicates per strain). Optical density was measured at 620 nm.

IAA synthesis was measured according to Ambrosini & Passaglia (2017), with some modifications. Bacteria were grown overnight in 3 mL of LB broth supplemented with 2.5 mM Tryptophan and then centrifuged at maximum speed for 10 minutes. Precipitated bacteria were washed in 3 mL of Tris-HCl 0.1 M, pH 7.6. After washing, bacteria were suspended in 1 mL of the same buffer, and OD600 was calibrated to 0.1. Afterwards, 30 µl of diluted bacteria were inoculated in 3 mL of LB + Tryptophan media, and grown for 2 days, at 28°C and 180 rpm. Then, bacterial cultures were centrifuged, and 100 µL of

supernatant was added to 100 µL of Salkowski reagent in a 96-well plate (three technical replicates per strain). Samples rested for 30 minutes in dark conditions, and optical density was measured at 535 nm.

The assessment of ACCd activity was performed according to Ambrosini & Passaglia (2017). Briefly, bacteria were grown in KB broth for 48 h, at 28°C and 180 rpm. Then, 1 mL of the bacterial culture was transferred to a microcentrifuge tube and centrifuged for 10 minutes at 5400g. Bacterial pellets were resuspended and washed, a total of three times, in 1 mL of 0.85% NaCl solution. Ultimately, 2 µl of each bacterial suspension was added to DF salts agar plates, both with and without ACC. Petri plates were incubated for 7 days, at 28°C, and the estimation of the use of ACC as a nitrogen source was evaluated through a comparison of growth between plates. Strains that grow in both media are positive for ACC deamination.

The abovementioned protocols allowed the selection of the two best bacteria to be analysed as a PGP. This selection was not solely based on single-trait maximisation, but on functional complementarity for drought mitigation. Also, to further assess each bacterium's potential, a data normalisation and consequent Weighted Score was calculated, so that the stronger ones could be selected. Data normalisation was performed on GraphPad PRISM 9.0.0. The Weighted Score was calculated based on the following equation:

$$WS = NSID + NPS + NIAA + NACCd$$

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in which NSID is the normalised value of the siderophore production results, NPS is the normalised value of the phosphate solubilisation results, NIAA normalised value of the IAA synthesis results, and NACCd is the normalised value of the ACCd synthesis results.

2.4 In-vivo assay of bacteria plant-growth promotion potential

The two best bacteria were selected to be analysed as a PGP solution in 2-year-old potted grapevines, from the 'Touriga-Nacional' cultivar, with the R99 rootstock. This assay was conducted in a greenhouse, with controlled temperature and relative humidity conditions, and was designed to have a total of six treatments, which consisted of: Bacteria 1; Bacteria 2; and Control (no bacteria supplementation), in both 80% and 10% field capacity (FC) irrigation. Each treatment consisted of five biological replicates (30 plants total). Grapevines were fully irrigated at T0 (22nd of May), weighted, 80% and 10% of the weight loss was calculated weekly, and irrigation was made accordingly.

Bacterial solutions were grown in LB broth for two days, centrifuged, resuspended with autoclaved dH2O, and then OD600 was calibrated to 0.1. Finally, 50 mL of the bacterial solution was prepared with autoclaved dH2O and applied to the respective pots (application at the surface, near the grapevine trunk) at three time points: 22nd May (after irrigation), 29th May, and 5th June. Control-treatment grapevines were treated with autoclaved dH_2O. Sampling and physiology measurements were performed on 7th

August, when five leaves per plant were collected, macerated in liquid nitrogen, and stored at -80℃ until needed. Enzymatic and photosynthetic activity, along with oxidative stress measurements, were performed, as each grapevine was considered a biological replicate, and three technical replicates of each were used. Chlorophylls a and b, carotenoids, and anthocyanins were quantified according to Sims & Gamon (2002). Briefly, chlorophyll a (Chl a), chlorophyll b (Chl b), carotenoids (Car), and anthocyanins (Ant) were extracted from frozen leaf powders in acetone: 50 mM Tris-HCl pH 7.8 buffer (80:20, v/v). Absorbance at 470, 537, 647, and 663 nm was read (FLUOstar Omega, BMG LABTECH). Results are expressed as mg/g FM. Total Soluble and Total Insoluble Sugars were quantified using the anthrone method, as described in Dias et al., 2018. Hydrogen peroxide (H_2O_2) and superoxide ions (O_2^-), as well as the quantification of Total Soluble Proteins, APx, and GPx, were assessed according to Araújo et al. (2021). Physiology measurements were collected before sampling using the Opti-Sciences OS30p+ fluorometer and the LI-COR LI-6800 (LI-COR Biosciences, Nebraska, USA). These measurements were taken around noon on fully expanded leaves (one per grapevine), and data regarding stomatal status, gas exchange, photosynthesis, and its products were collected.

2.5 Soil sampling and microbial community identification

To detect the presence of the inoculated bacteria on the soil of potted grapevines, one soil sample per grapevine was collected (10 cm depth), on August 6th, 2024. DNA extraction was performed with the E.Z.N.A. ® Soil DNA Kit (Omega Bio-tek, Inc., GA, USA) and DNA integrity was assessed with electrophoresis on 1% agarose gel and quantified using LVis Plate and the FLUOstar® Omega Multiplate reader (BMG Labtech, Germany). The six DNA samples per vineyard (four vineyards total) and time point (four time points total) were pooled and high molecular weight DNA aliquots with 260/280 and 260/230 ratios ranging between 1.8–2.0 were then sent to Novogene Corporation Inc. (Cambridge, UK) for library preparation and sequencing.

Bacterial DNA was amplified using primers targeting the 16S ribosomal RNA (rRNA) V3-V4 hypervariable region: CCTAYGGGRBGCASCAG (341F) and GGACTACNNGGGTATCTAAT (806R) (Novogene Corporation Inc., Cambridge, UK). Amplicon generation was done using 15 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, USA); 0.2 µM of forward and reverse primers, and 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98 ℃ for 1 min, followed by 30 cycles of denaturation at 98 ℃ for 10 s, annealing at 50℃ for 30 s, and elongation at 72℃ for 30 s and 72℃ for 5 min. The PCR products of proper size were selected through 2% agarose gel electrophoresis, pooled, end-repaired, A-tailed, and further ligated with Illumina adapters. Finally, libraries were sequenced using Sequencing by Synthesis (SBS) technology on a paired-end Illumina NovaSeq 6000 platform to generate 2 × 250 bp paired-end raw reads. The library was checked with Qubit and real-time PCR for quantification, while a bioanalyzer was used for size distribution detection. Quantified libraries were pooled and purified with the Universal DNA Purification Kit (TianGen, China).

Paired-end reads were demultiplexed and assigned to each sample based on their unique barcodes and truncated by removing the barcode and primer sequences. Clean reads were then analysed using One

Codex (https://app.onecodex.com), and taxonomic data of the samples were obtained and filtered to the family and/or genus of interest for this work.

2.6 Statistical analysis

Statistical analysis and consequent graphics were performed using GraphPad PRISM 9.0.0. The results of each protocol were subjected to a Two-Way ANOVA and, consequently, an HSD—Tukey test. Because some significant results in the Two-Way ANOVA were not shown in the Tukey test, an LSD—Fisher test was also conducted. For the principal component analysis (PCA biplot) with confidence ellipses (≤ 95%), Python 3 was used with the packages Pandas, Scikit-learn, Matplotlib and Numpy.

3. Results

3.1 Biochemical screening

Given the large number of collected samples and isolated strains (a total of 400 isolates were initially obtained), a preliminary screening of the bacterial collection was performed as described in Section 2.2. Only the bacteria that showed positive results in both protocols (qualitative siderophore production and phosphate solubilisation) were selected, corresponding to nine isolates: B009, B011, B105, B140, B258, B263 and B264. The second screening included siderophore production, phosphate solubilisation, IAA synthesis and ACC deaminase activity analyses. Regarding the abovementioned protocols, the obtained results are available in Table 1.

Table 1
Biochemical activity regarding siderophore production, phosphate solubilisation, IAA synthesis, and ACCd activity of the bacterial strains; 1 means “growth”, 0 means “no growth”.

Isolate	Siderophore production (halo cm)	Phosphate solubilisation (µg/mL)	IAA synthesis (µg/mL)	ACC deaminase
B009	2.0	194.87	3.27	0
B011	2.0	172.56	27.11	0
B105	1.1	185.99	6.84	1
B140	1.9	172.59	86.06	1
B258	1.5	208.85	5.81	1
B263	2.1	219.87	10.64	0
B264	1.8	173.54	7.41	1

As aforementioned, all the selected isolates can produce siderophores. However, siderophore production quantification revealed that all isolates produce a halo of similar size, with a notably lower production for isolate B105. Phosphate solubilisation results were positive in all strains, as was IAA synthesis, however,

B140 has far superior IAA synthesis capacity than all other isolates. Regarding ACC deaminase, results were more divided, with B105, 140, 258 and 264 showing a capacity to break down ACC.

These bacterial isolates were also taxonomically identified, at the species level, as revealed in Table 2:

Table 2
Bacterial taxonomic identification with 16S rRNA sequencing.

Isolate	Taxonomy	Identity percentage
B009	*Serratia marcescens*	98.81
B011	*Serratia marcescens*	99.08
B105	*Bacillus mycoides*	99.12
B140	*Myroides odoratimimus*	98.51
B258	*Arthrobacter nicotinovorans*	99.93
B263	*Serratia marcescens*	98.76
B264	*Serratia marcescens*	98.69

Acknowledging the results in Table 1, the strain taxonomical identification in Table 2, and the selection criteria defined in the Materials and Methods section, Table 3 shows the Weighted Score (WS) of the results obtained by the analysed bacteria:

Table 3
Normalised data and corresponding Weight Score of the biochemical characteristics of the bacteria in analysis; NSID – Normalised siderophore production; NPS – Normalised phosphate solubilization; NIAA – Normalised indolacetic acid synthesis; NACCd – Normalised ACCd production.

Bacteria	NSID	NPS	NIAA	NACCd	WS
B009	0.818	0.472	0.000	0	**1.290**
B011	0.818	0.000	0.288	0	**1.106**
B105	0.000	0.284	0.043	1	**1.327**
B140	0.727	0.001	1.000	1	**2.728**
B258	0.364	0.767	0.031	1	**2.161**
B263	0.909	1.000	0.089	0	**1.998**
B264	0.636	0.021	0.050	1	**1.707**

B140 and B258, *Myroides odoratimimus* (Flavobacteriaceae family) and *Arthrobacter nicotinovorans* (Micrococcaceae family), respectively, were selected to proceed with the work, being the two with the

highest weight score. These bacteria were two of the ones that had positive results in all biochemical screenings but stood out in different protocols: *Myroides odoratimimus* stood out in IAA synthesis (86.05 µg/mL), whilst *Arthrobacter nicotinovorans* stood out in phosphate solubilisation (208.85 µg/mL).

3.2 PGP potential assessment on potted grapevines

The two selected bacteria were grown in LB broth, and solutions with dH_2O were prepared and applied to potted grapevines, as described in section 2.4. After the execution of the abovementioned protocols, the following results were obtained (Table 4):

Table 4

Two-way ANOVAs of the physiology protocols. Statistically significant impacts are highlighted in bold and with asterisk marks; Chl a – Chlorophyll a; Chl b – Chlorophyll b; CAR – Carotenoids; ANT – Anthocyanins; PI – Performance Index; gs – Stomatal Conductance; A – CO_2 Fixation; TSS – Total Soluble Sugars; TIS – Total Insoluble Sugars; A/gs – CO_2 Fixation Efficiency; TPC – Total Phenols Content; E – Transpiration; H_2O_2 – Hydrogen Peroxide; O_2^- – Superoxide ion; TSP – Total Soluble Proteins; APx – Ascorbate Peroxidase; SOD – Superoxide Dismutase; GPx – Guaiacol Peroxidase.

Two-Way ANOVA			
Protocol	**Irrigation**	**Bacteria**	**Interaction**
Chl a	**0.0017****	0.68	0.2788
Chl b	**0.0255***	0.3668	0.2972
CAR	0.2554	**0.0353***	0.3296
ANT	0.3671	0.9649	0.5773
PI	0.0557	**0.0426***	0.7681
gs	**< 0.0001****	0.1098	**0.0105***
A	**0.0015****	0.8742	0.1729
TSS	**0.0184***	0.4126	0.1676
TIS	**0.0165***	0.2142	0.6607
A/gs	0.6741	0.4419	0.8596
TPC	0.9307	0.7905	0.5642
E	**0.0002*****	**0.0434***	0.0505
H_2O_2	0.586	0.5929	**0.0150***
O_2^-	**0.0179***	0.683	0.7642
TSP	0.0651	0.7647	0.6691
APx	0.8388	0.1508	0.2134
SOD	**< 0.0001****	**0.0009*****	0.1011
GPx	0.6944	0.5123	0.397

Acknowledging the results of the Two-Way ANOVA shown in Table 4, the most influential factor concerning the grapevines' physiology was "Irrigation". It has impacted Chl a, Chl b, gs, A, TSS, TIS, E, O_2^-, and SOD. The "Bacteria" factor also had some impact on a few physiological parameters, such as CAR, PI, E, and SOD. Finally, a significant interaction between both factors was observed in gs, whilst H_2O_2. ANT, TPC, TSP, APx, and GPx were not influenced.

The boxplots in Figs. 2 and 3 illustrate the statistical significance of the data when subjected to the HSD–Tukey multiple comparison test. Regarding the boxplots in Fig. 2, despite the statistical significance attributed to the "Irrigation" factor on Chl a and b, Tukey did not identify statistically significant differences between specific groups. However, a notable difference is observed between B258_80 and B258_10. Overall, the Two-Way ANOVA indicates that both pigments are enhanced by water availability, which becomes more apparent if an LSD – Fisher test is performed instead of Tukey (see Supplementary Data – 2).

Also, simple One-Way ANOVAs between "Bacteria" factor suggest that, even though not significantly, it seems that Chl a and Chl b have higher contents in both bacteria treatments than CTRL in 80% FC grapevines (See Supplementary Data – 3). Regarding CAR, the Two-Way ANOVA indicated that the "Bacteria" factor statistically impacts the results. However, the Tukey analysis did not indicate which groups are statistically different, even though it seems (boxplot of Fig. 2) that B140 is promoting carotenoid synthesis, mainly in 80% FC grapevines, compared to CTRL. The results of the Fisher test show that both irrigation regimes under the B140 application had significantly higher CAR content than both CTRL irrigation regimes. Moreover, regarding A, gs, and E, similar patterns are observed. In these parameters, "irrigation" had high impacts, as it seems that, in conditions of high water availability, CTRL grapevines performed better than both bacteria groups (mainly observed in gs, where CTRL_80 had statistically higher results than B140_80). However, in conditions of drought stress, the opposite seems to happen (supported by the significant result of the "Irrigation x Bacteria" interaction of the Two-Way ANOVA regarding gs), as no statistically significant differences are noticed comparing both irrigation regimes inside both bacteria groups, but in the CTRL group there are significant losses of activity in these three parameters (also observed in the Fisher analysis in Supplementary Data – 2). These insights are extremely relevant to understanding photosynthetic dynamics in these grapevine groups.

Regarding Total Soluble and Total Insoluble Sugars, despite the statistically significant results indicated by the Two-Way ANOVA for the "Irrigation" factor, the Tukey test did not allow any conclusion on which groups promoted or restrained them. However, grapevines with hydric comfort apparently performed better in sugar synthesis than those exposed to drought stress, as the Two-Way ANOVA suggests. It also seems that the CTRL grapevines of 10% FC had statistically lower TIS content than the 80% FC of CTRL and both irrigation regimes of both bacteria, which is confirmed by the Fisher test. Regarding H_2O_2 and SOD, the Two-Way ANOVA pinpointed statistical significance for the interaction between factors, and for both factors, respectively. In H_2O_2, it seems that the grapevines inoculated with both bacteria had no variations with the variability in water availability, whilst in CTRL, it seems that H_2O_2 content increased in

10% FC treatment, compared to 80% FC (observed in Fisher but not in Tukey). Once again, it seems that, in 10% FC grapevines, the CTRL group is more impacted by water availability than the other groups. The boxplot of SOD also suggests this, as CTRL 10% FC has the highest SOD content among all treatments, even though being less evident than the example of H_2O_2. Total phenols and soluble protein contents were unaltered by any factor.

Regarding the many analysed parameters, a Principal Component Analysis (PCA) is crucial to understanding the grapevines' dynamics and status. Therefore, the PCA was performed, and the results are shown in Fig. 4. This PCA shows the 2 major PCs of the analysis, as PC1 explains 23.5% of the variability of the results, whilst PC2 explains 14.53% of it. In this case, PC1 seems to be strongly associated with photosynthetic and metabolic performance, and PC2 is associated with the type of antioxidant response of the grapevines to the induced drought stress.

By analysing the results on the PCA, it is clear that, in 10% FC grapevines, the CTRL group was the one that experienced a higher oxidative stress, suggesting that the treatments with B140 and B258 reduced the impact of the drought stress in their groups' grapevines. It is also worth noting that, in the case of 10% FC groups, CTRL seems to have a different antioxidant response to stress, as it is mainly focused on the SOD pathway, whilst the B258 group is more focused on the GPx and TPC pathways. In the case of B140 10% FC grapevines, their stress response seems to be more balanced between all the analysed parameters. Furthermore, the PCA suggests that the grapevines inoculated with both bacteria maintain higher photosynthetic activity than the CTRL group.

Regarding the 80% FC groups, it seems that B140 and B258 are associated with higher photosynthetic rate, CO_2 assimilation, stomatal conductance and transpiration, suggesting improvements in their performances, namely in converting light energy into chemical energy. The increases of gs and E could indicate better stomatal regulation induced by the bacteria, and, consequently, higher CO_2 assimilation, and TSS and TIS production. The increase in the synthesis of photosynthetic pigments, namely Chl a, Chl b, and CAR, is also noted on the grapevines treated with both bacteria, promoting higher light absorption.

Finally, it is noted that certain photosynthetic parameters, such as A, gs, E, TIS, and TSS, are strongly interlinked, suggesting that grapevines with higher photosynthetic rates also exhibit greater transpiration and solute content. Conversely, some oxidative stress parameters behave oppositely to the photosynthetic ones, with the most apparent example being SOD, indicating that grapevines experiencing higher oxidative stress are limiting their photosynthetic activity.

3.3 Assessment of rhizosphere colonisation

The metagenomic profiling of the bacterial communities present in the rhizosphere of potted grapevines revealed distinct colonisation patterns between the two bacteria. These results showed a 2.6-fold increase in *Arthrobacter* read count in soil samples from B258-treated 10% FC potted grapevines, compared to those obtained from CTRL_10 (Fig. 5). However, the presence of *Myroides* bacteria was not

detected, despite the slight upward (but non-significant, as high variability in control samples is observed) trend of the Flavobacteriaceae family group, as observed in Fig. 5.

4. Discussion

Climate change threatens viticulture and its products, with expected organoleptic characteristics, productivity and yield modifications, and ultimately loss of *terroir* identity. This could endanger the socio-economic dynamics of the regions with strong connections to this activity, potentially harming employment in rural areas (Fraga & Santos, 2021).

Strategies must be devised to alleviate the effects of climate change on viticulture, particularly by ensuring that grapevines can maintain their productivity and the quality of their products, even when temperatures rise and water availability diminishes. The application of autochthonous PGP bacteria may be one of the most interesting strategies, as they are described to enhance plants' potential due to their biofertilising and/or biostimulating characteristics, whilst being adapted to the local environment and respective bacterial community (Ambrosini & Passaglia, 2017; Ipek & Eşitken, 2017). Autochthonous PGP application is a low-budget, sustainable, non-invasive solution, unlike irrigation or phytochemical applications, making it an even more interesting strategy to explore (Haskett et al., 2021; Yadav, 2017). In fact, the currently used strategies need to be periodic in climate change hotspots, as both water and phytodchemicals are rapidly consumed, and end up being costly and unsustainable, as there is a higher need for accurate and responsible water and soil management. Among the bacteria in this study, DDR-authoctone *Myroides odoratimimus* and *Arthrobacter nicotinovorans*, B140 and B258, respectively, were evaluated as a PGP treatment in grapevines, both in hydric comfort and drought stress, as both demonstrated noteworthy results in the previous biochemical assays.

The results of the assessment of the PGP potential of *M. odoratimimus* and *A. nicotinovorans* showed that these bacteria had some influence on the physiological performance of the potted grapevines. Firstly, it seems that B140 favoured carotenoid synthesis (25.7%) in 80% FC grapevines, and despite not yielding significant results, both bacteria appear to have promoted the concentration of both Chl a (13.2% for B140 and 17.9% for B258) and b (15.8% for B140 and 17.5% for B258) in 80% FC (Supplementary Data – 7), which could be attributed to their capacity to produce siderophores, reported to enhance the uptake of several nutrients. Some of them are Fe and Zn, which are cofactors and activators to enzymes, respectively, participating in the synthesis chains of chlorophylls a and b, and contributing to grapevine growth (Bhatia et al., 2025; Bruno et al., 2020; Erdogan et al., 2018; Zhu et al., 2025). Siderophores are also linked to enhancing the grapevine's tolerance to stress, as they chelate heavy metals and reduce oxidative stress levels, decreasing H_2O_2 and increasing the activity of antioxidant enzymes, such as SOD, CAT, and APx (Funes Pinter et al., 2017). The chelation of Fe is beneficial in two ways, as it not only facilitates the absorption of plants but also limits iron availability for pathogens, suppressing their development (Miethke & Marahiel, 2007).

The impacts of both bacteria on photosynthetic performance were assessed, and similar patterns were observed in stomatal conductance, CO_2 fixation, and transpiration rate. These results, in which it can be observed an important role of both *M. odoratimimus* and *A. nicotinovorans* in mitigating drought stress impact on photosynthesis, could be due not only to the abovementioned role of siderophores, but also to both bacteria's capacity to synthesise ACCd, which, by scavenging the ethylene precursor ACC, limits ethylene synthesis and, consequently, its role in chlorophyll degradation and stomata closure, and overall oxidative stress, which is exactly what is suggested by the results (Glick, 2005; Shahid et al., 2023).

However, despite having some similar characteristics, namely regarding siderophore production and ACCd synthesis, it seems that these bacteria activated different stress mitigation mechanisms in the grapevines, as observed in the PCA. *M. odoratimimus* appears to have promoted a more balanced response in grapevines (PCA), but stood out as a high IAA producer, which stimulates cellular proliferation and elongation, and thereby fosters root and aerial part growth (Bruno et al., 2020; Patten & Glick, 2002). As more developed roots can explore higher soil volumes, the promotion of root development directly improves water and nutrient absorption, mitigating water shortage impacts (Aguilar et al., 2021; Rolli et al., 2017). IAA also promotes pigment biosynthesis, namely chlorophylls (Ma et al., 2022), increasing the capacity to absorb light and enhancing the grapevine's capacity to maintain a robust photosynthetic rate, as observed in the results. The impact of *M. odoratimimus*, by enhancing IAA, whilst also synthesising ACCd, seems to prevent drought stress and to improve photosynthetic rate (41.9% higher than CTRL 10% FC), not needing to activate the antioxidant pathways, as H_2O_2 levels in 10% FC grapevines treated with this bacterium were significantly lower than the ones treated with *A. nicotinovorans* and the CTRL group (–19.5% and − 35.0%, respectively), with no increases in the antioxidant machinery (Supplementary Data – 2). By understanding this role of IAA, *M. odoratimimus* application can be considered a more long-term, sustainable one, as it promotes a structural, more resilient growth, better preparing grapevines for climate change perspectives.

A. nicotinovorans seems to have a distinct approach. Being a high phosphate solubiliser, it increases PO_4^{2-} uptake, and improves phosphorilation and energy transfer, which could activate the antioxidant pathway, as suggested by the PCA (B258_10 seems to be highly modulated by GPx and TPC, and to have a higher APx activity (70.1% higher than CTRL) in 10% FC grapevines – Supplementary Data – 8). It is well known that under drought stress, the balance of reactive oxygen species (ROS) in plants is disrupted (Miller et al., 2010). APx is recognised for its role in alleviating oxidative stress by converting the harmful ROS H_2O_2 into water molecules (Das & Roychoudhury, 2014). Therefore, *A. nicotinovorans*, by enhancing APx concentration, might be able to mitigate the oxidative stress induced by drought. Indeed, B258-treated grapevines displayed a lower H_2O_2 concentration in drought-stress conditions than CTRL (–19.3%), probably due to the APx increase. This activity, associated with the capacity of *A. nicotinovorans* to produce ACCd, decreases ethylene and its stress effects, and maintains the photosynthetic capacity (41.9% higher than CTRL 10% FC grapevines) and stomatal conductance (39.8% higher than CTRL 10% FC grapevines), as observed in our results (Supplementary Data – 2). The stress-mitigating effects of *A.*

nicotinovorans seem to be a more immediate, short-term solution, protecting the grapevine from drought stress, but not in a structural manner, such as observed with *M. odoratimimus.*

Contrary to expectations, neither bacterial strain statistically promoted total soluble sugar content under both irrigation conditions. However, in hydric comfort grapevines, the average values of TSS treated with both bacteria are higher than CTRL, and statistical significance is prejudiced by the high standard deviations (Supplementary Data – 4). In contrast, inoculation with both bacteria might be able to mitigate drought-induced effects, potentially by enhancing carbon storage in the form of insoluble sugars (67.2% higher in B140-treated 10% FC grapevines, and 62.0% higher in B258-treated 10% FC grapevines). It seems that there is a direct correlation between TIS and A, gs, and E, suggesting that it was the maintenance of these photosynthetic indices, promoted by both bacteria, that allowed the continuation of TIS accumulation. These insoluble sugars serve as a carbon reserve, which the plant can utilise to maintain a carbon supply and increase drought resistance (Piper, 2011; Regier et al., 2009), with the added benefit that starch can be readily degraded into soluble sugars when needed (Dietze et al., 2014), such as recovering from drought stress or preparing for the subsequent productive cycle. Together, soluble and insoluble sugars not only act as carbon and energy reserves but might also play a crucial role in maintaining proper osmotic balance within the plant, the capacity to keep producing defence secondary metabolites, such as APx, and the possibility to continue radicular growth, which is fundamental in drought stress scenarios (Dietze et al., 2014). Therefore, both bacteria showed they can regulate the grapevines' carbon metabolism by maintaining their conditions to develop rooting systems (through IAA) and/or mitigating oxidative stress (through phosphate solubilisation and ACCd production). With this, it was possible to maintain the photosynthetic activity and, consequently, carbon storage, which was crucial for the survivability of the grapevines in water-shortage conditions. This characteristic is another differentiating factor from the current suggested approaches to mitigate the impacts of drought stress.

The physiological benefits of the inoculation of *A. nicotinovorans* were evident and directly linked to the 2.6-fold enrichment of *Arthrobacter* in the rhizospheric bacterial community. However, we were not able to demonstrate the presence of *M. odoratimimus* in the rhizosphere of the grapevines treated with B140, even though significant structural adaptations were shown (maintained photosynthetic capacity and TIS accumulation in drought stress scenario). These results suggest that *M. odoratimimus* could have had a significant impact on the grapevine through an early-stage priming of the root system, having a biostimulant effect, mainly mediated by its high IAA synthesis, before suffering from the drought scenario. This pinpoints that some PGP, which have the capacity to improve grapevines' conditions, could not require high population density at the end of the crop cycle, particularly in the case of *M. odoratimimus*, as it employs hormonal modulation strategies. Another hypothesis for the absence of the detection of *M. odoratimimus* on soil samples could be its characteristic as a facultative endophyte, which is typical of high IAA-producing bacteria, as it modulates root architecture to facilitate colonisation, escaping detection on bulk rhizosphere soil analysis (Sudharsan and Kannan, 2025; Zhang et al., 2022).

The insights provided by the results in this work indicate that the application of *M. odoratimimus* and *A. nicotinovorans* provides different resources and stimulates different mechanisms for grapevines to cope with drought stress. With this, we can presume that their roles could be complementary, possibly increasing even further the grapevine's resistance to drought when applied as a consortium, as it would grant a wider protection. However, this needs to be assessed through new plant assays, after the two bacteria's compatibility is confirmed. Another point needing to be explored is the testing of these results in a field trial, which is fundamental to securely assure that these two bacteria are, in fact, a real solution for farmers in the Douro Demarcated Region.

5. Conclusion

Climate change, with its critical threat to the viticultural identity of the Douro Demarcated Region, demands the development of innovative, ecological, and sustainable strategies, that go beyond traditional water and soil management. As viticulture is one of the most important sectors in agriculture, namely due to its relevance to the Douro Demarcated Region's socio-economic and cultural dynamics, the exploration of autochthonous PGP bacteria becomes a fundamental strategy, and this study demonstrates that their application is not merely a supplementary strategy, but a decisive one, regarding drought adaptation strategies.

Our findings reveal a functional dichotomy in the mechanisms of action of both *M. odoratimimus* and *A. nicotinovorans* in stress mitigation. These PGPs had an impact on the grapevines' physiology, mainly by acting as a preventive structural primer, in the case of *M. odoratimimus*, or as a reactive defender, in the case of *A. nicotinovorans*, which maintained energy storage through TIS accumulation, unlike in grapevines subjected to drought stress with no bacterial treatment. These results could be directly linked to their PGP characteristics, as *M. odoratimimus* preventive action is modulated by high IAA synthesis and complemented by siderophore production and ACCd synthesis, inducing photosynthetic stability and preventing the grapevines from oxidative stress. *A. nicotinovorans* reactive defence, promoted by high phosphate solubilisation, ACCd synthesis and siderophore production, empowers the grapevines' tolerance to drought stress, through increased antioxidant enzymatic activity.

The different mechanisms of action of the two PGPs suggest that they could be complementary, and that effective drought mitigation in viticulture may require a multi-layered approach, lying in tailored bacterial consortia. Therefore, further studies are needed to determine if a consortium of the preventive mechanisms of *M. odoratimimus* and the reactive ones of *A. nicotinovorans* can be more impactful in mitigating drought stress, and how a treatment combining these two bacteria would influence grapevines in field conditions. With this combination of characteristics, we could develop a holistic bio-strategy capable of preserving the productivity and the unique *terroir* of the DDR, even with the prospects of climate change.

Declarations

Competing Interests

The authors declare that there are no competing interests regarding this work.

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Author Contribution

J.P. prepared the assays, conducted it, collected data, analysed results, and wrote the manuscript.P.O.P. and J.O.F. collected data and wrote the manuscript.R.F. assisted in the conduction of the assays and collected data.S.M. wrote the manuscript.L.T.D. collected data, supervised, and reviewed the manuscript.C.S. obtained funding, supervised, and reviewed the manuscript.

Data Availability

The data will be made available at request.

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Figures

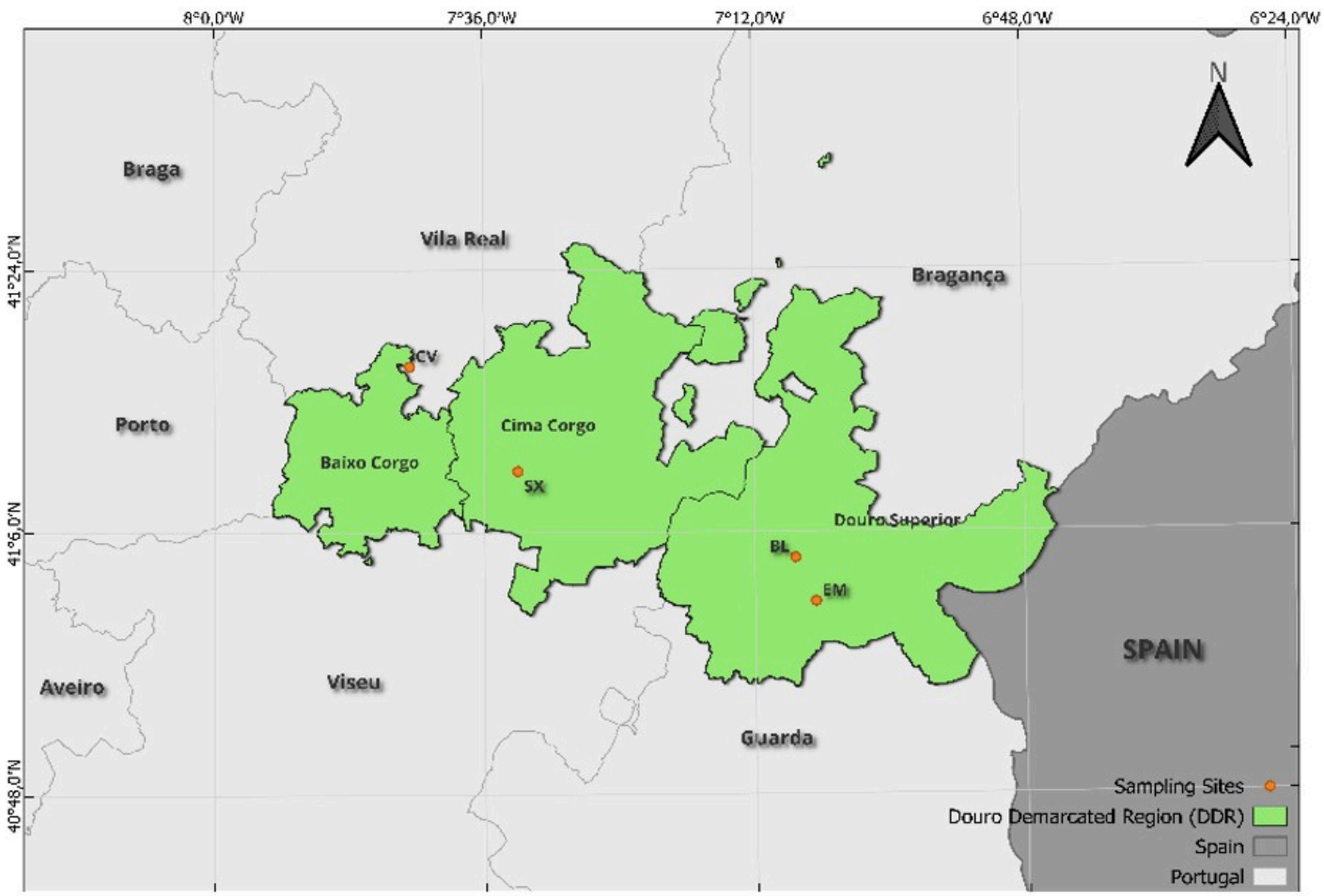


Figure 1

Sampling sites/vineyards location in the Douro Demarcated Region; CV - Quinta do Cavernelho; SX - Quinta do Seixo; BL - Quinta Belém; EM - Quinta da Ervamoira.

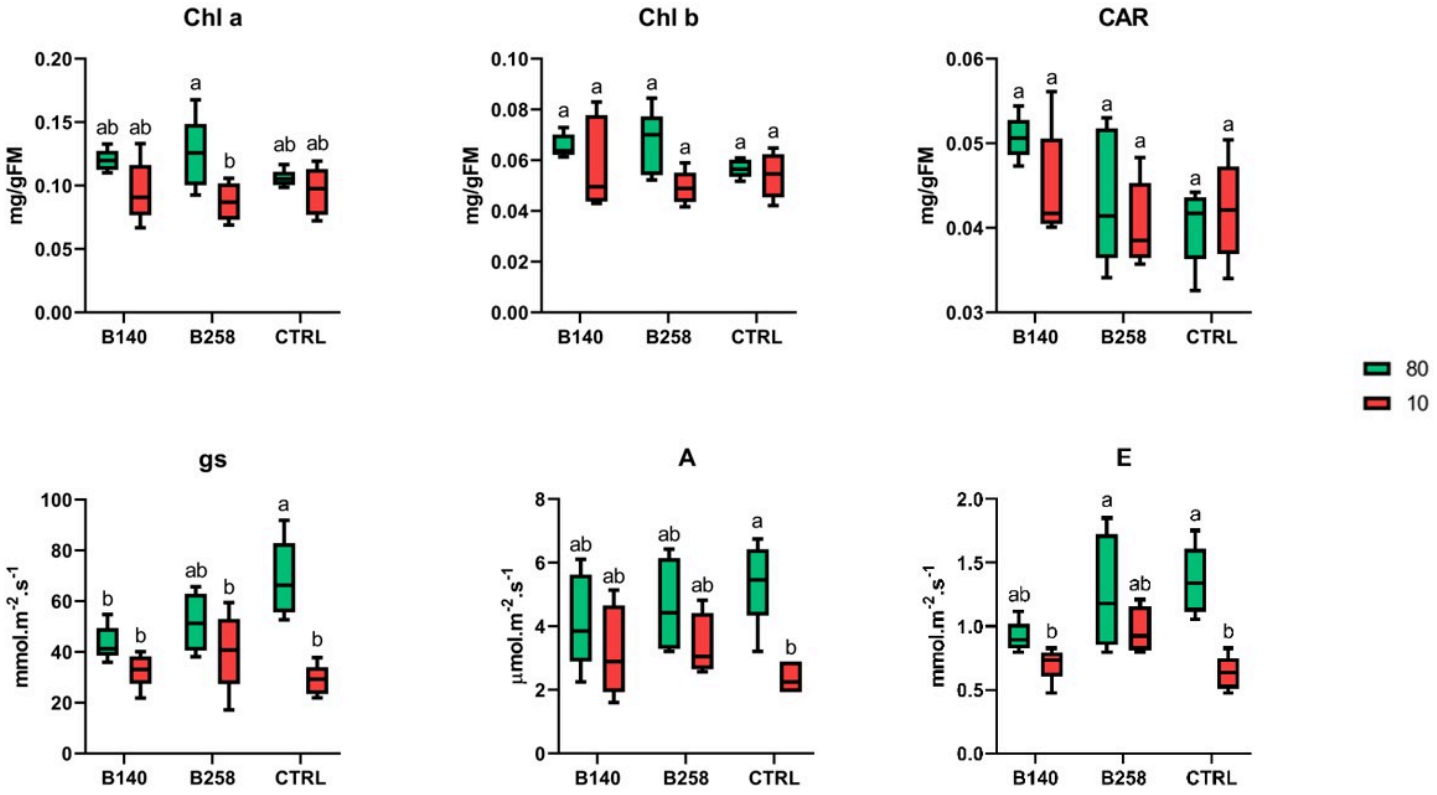


Figure 2

Boxplot of the results for HSD – Tukey of each analysed physiological parameter; Chl a – Chlorophyll a; Chl b – Chlorophyll b; CAR – Carotenoids; gs – Stomatal Conductance; A – CO_2 Fixation; E – Transpiration Rate; 80 – 80% field capacity irrigation; 10 – 10% field capacity irrigation; Different letters mean statistically different results.

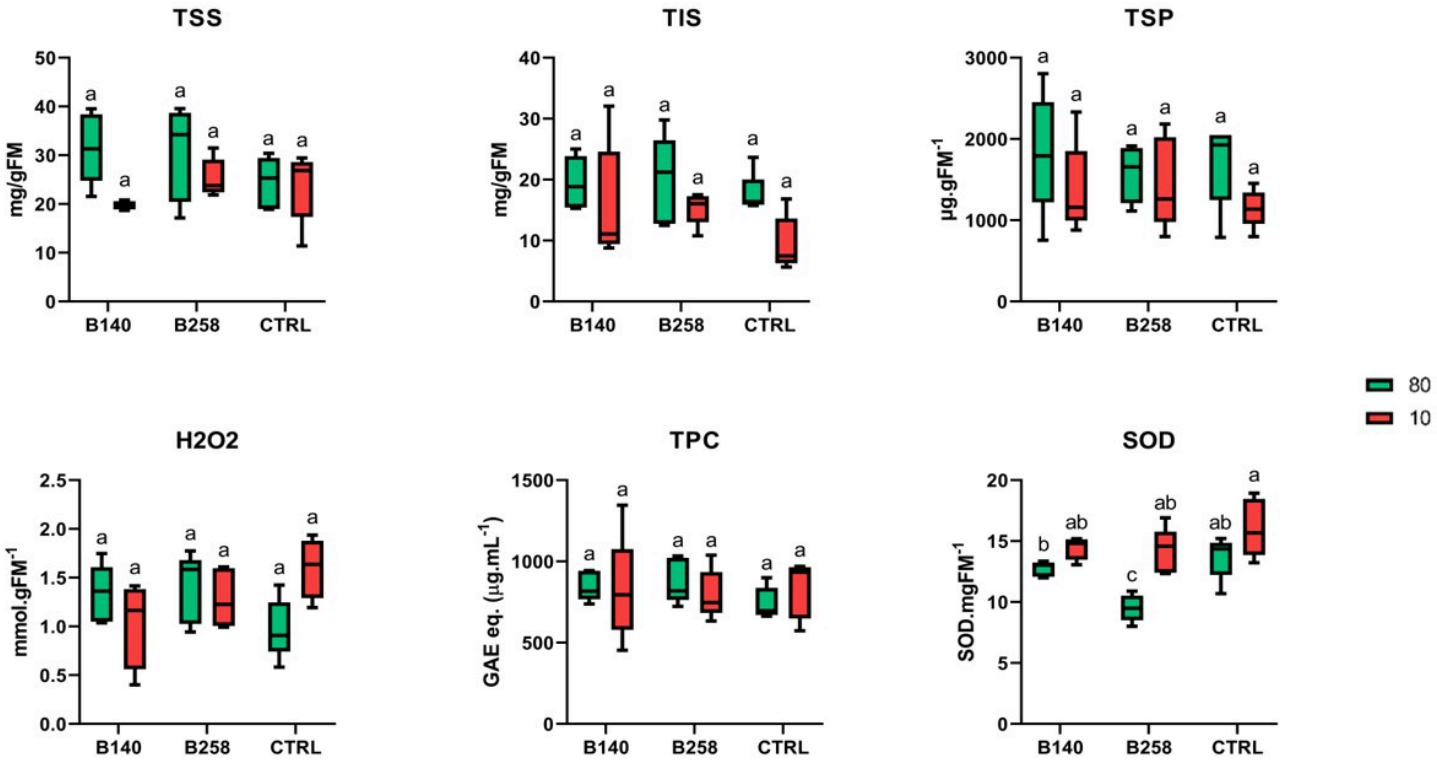


Figure 3

Boxplot of the results for HSD – Tukey of each analysed physiological parameter; H_2O_2 – Hydrogen peroxide; SOD – Superoxide dismutase; 80 – 80% field capacity irrigation; 10 – 10% field capacity irrigation; Different letters mean statistically different results.

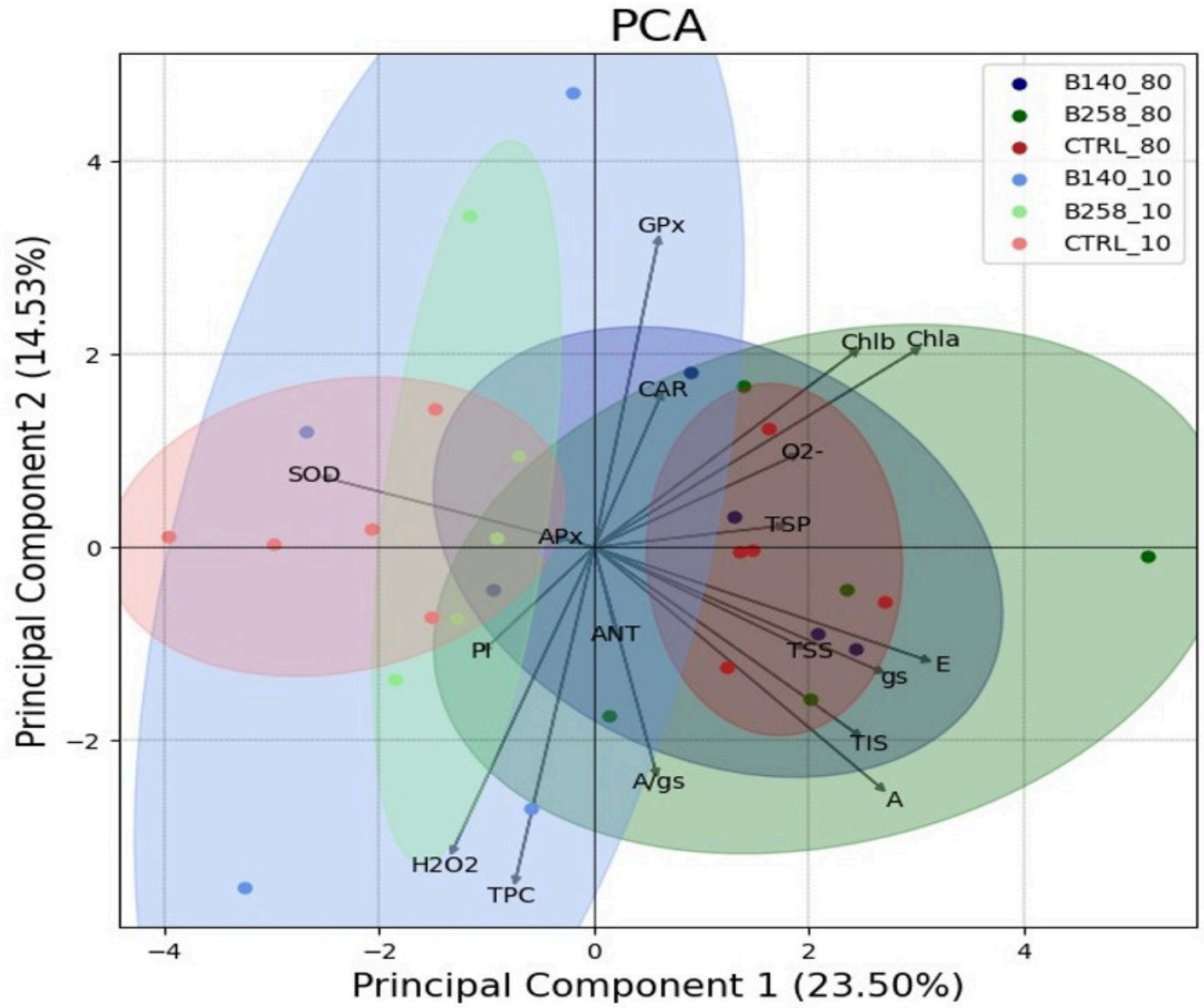


Figure 4

Principal Component Analysis of the analysed parameters in the different treatments of the assay. B140 – Myroides odoratimimus; B258 Arthrobacter nicotinovorans; CTRL – Control plants; Performance index (PI), Photosynthetic Rate (A), CO_2 fixation efficiency (A/gs), Stomatal Conductance (gs) Total Soluble Proteins (TSP), Guaiacol Peroxidase (GPx), Ascorbate Peroxidase (APx), Superoxide Dismutase (SOD), Total Phenols Content (TPC), Hydrogen Peroxide (H_2O_2), Superoxide ion (O_2^-), Transpiration rate (E), Total Soluble Sugars (TSS), and Total Insoluble Sugars (TIS)

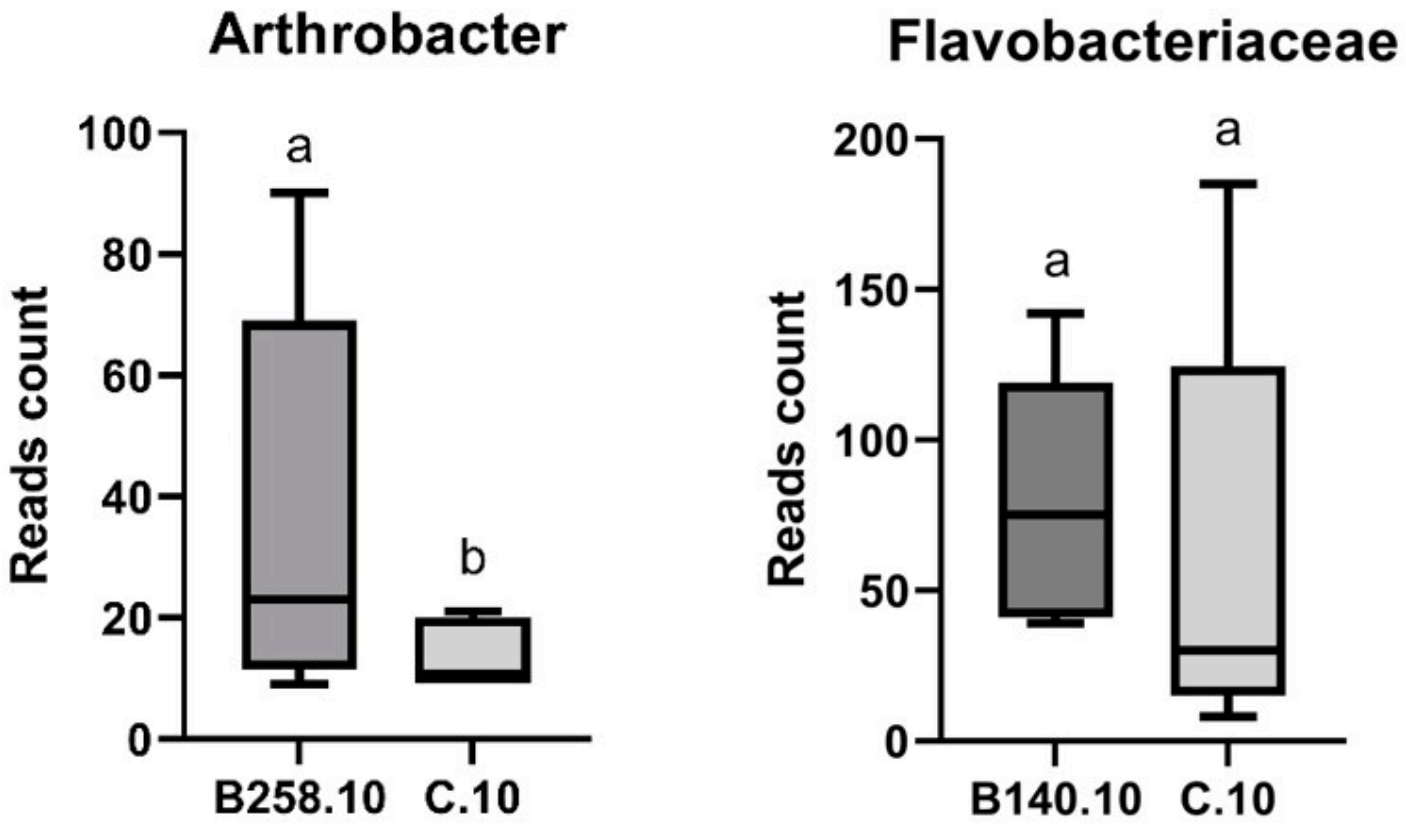


Figure 5

Relative abundance (reads count) of the Arthrobacter genus and of the Flavobacteriaceae family, in the respective treatments; B258.10 – group of 5 potted grapevines treated with B258 and subjected to drought stress; B140.10 – group of 5 potted grapevines treated with B140 and subjected to drought stress; C.10 – group of 5 potted grapevines with no bacterial treatments, subjected to drought stress.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryData.docx