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Biostimulant Effect of Commercial Rhizobacteria Formulation on the Growth of *Vitis vinifera* L.: Case of Optimal and Water Deficit Conditions

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Abstract: As climate change is an imminent threat to the environment and agriculture, there is an increasing need to find immediate solutions capable of compensating for water deficits even in semi-arid conditions. This study is focused on the evaluation of the vegetative growth of grapevine plants *Vitis vinifera* L., of the Greek variety "Debina" in a water deficit environment, with the application of two bacterial-based formulations: one with *Bacillus amyloliquefaciens* (strain QST 713) and one with *Sinorhizobium meliloti* (strain cepa B2352). The two formulations were tested under rational irrigation (100% of Available Water) and deficit irrigation (57% of AW). After 140 days, plant growth parameters, such as total plant growth length, leaf area, roots, shoots, and leaves dry biomass showed better performance on treatments with plant growth-promoting rhizobacteria (PGPR) formulations under either rational or deficit irrigation conditions. In addition, the metabolic response of the grapevine plants to the deficit irrigation stress, such as the total chlorophyll, leaf relative water, total phenolic, and proline content, proved to be enriched on the treatments with PGPR formulations during this experiment. The two formulations, in conditions of abiotic stress, achieved to almost compensate for the irrigation deficit, boosting the plant metabolism. This study reveals the need for further research on PGPR biostimulants, as this first trial of these formulations on grapevine could be significant in the case of water scarcity and climate change.

Keywords: biostimulants; PGPR; *Bacillus amyloliquefaciens*; *Sinorhizobium meliloti*; *Vitis vinifera*; Debina; irrigation deficit

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1. Introduction

Vitis vinifera L., (Vitales: Vitaceae) commonly known as grapevine, is one of the most important crops in Greece, as there are more than 60,000 ha, and it has been widely cultivated in the last centuries for its fruit, raisins, and wine [1]. The Greek variety "Debina" is indigenous to the viticultural zone of Zitsa, Ioannina (Epirus, Greece) at 600–700 m altitude and is used for winemaking. Grapevine is considered a drought-resilient species and its cultivation in a small percentage of water stress can improve the quality of berries and wine composition [2,3], although the vast climate change conditions of overheating and the continuous lack of water, are going to significantly damage vine production, yielding low-quality grapes [1]. An important impact of water deficit in grape cultivation is the alteration of gene expression that regulates the metabolic pathways that control the accumulation of secondary metabolites (flavonoids) that affect the quality characteristics of grape berries and wine flavor quality traits [4,5]. The diversity of metabolic responses to drought stress induced by deficit irrigation is common and depends on the genetic background of the variety [6]. Usually, the grapevine during the application of various water deficit irrigation fractions, presents higher concentrations of phenolic compounds [4,5,7,8]. Climate change conditions can affect grapevine cultivation as many studies and reviews mention [9–12].

Kizildeniz et al., 2015 [11] shows that drought combined with elevated temperatures can reduce grapevine performance. The combination of climate change factors that cause abiotic stress (elevated temperature and drought) drastically reduced vegetative growth, bunch fresh and dry weights in red and white Tempranillo grapevine cultivars [11].

An alternative aspect that may bring some solutions to the upcoming drought stress in various crops [13], including the grapevine, is expected to be microbiological formulations that can help plant defense to alleviate the harmful effects of abiotic stress, known as biostimulants [14,15]. Beneficial rhizobacteria have been studied for their positive effects on plant metabolism for a multitude of phytopathogens [15] while it appears that they also benefit plant growth and therefore they act as biostimulants [16] especially in abiotic stress conditions [17]. Also, some PGPR biostimulants can regulate the reactive oxygen species (ROS) levels in many plants under abiotic stress, resulting in increased proline and sugar levels [18,19]. The most commonly responsible modes of action by PGPR for enhancing plant growth under abiotic conditions, are 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, auxin production of cytokinins (CK), gibberellins (GB), indole acetic acid (IAA), and solubilization of phosphate and nitrogen fixation [18,19].

Bacillus amyloliquefaciens (Bacillales: Bacillaceae) is widely used and well-known PGPR for its pathogen-fighting potential on the roots of many plants and lately looming to have a biostimulant effect on many cultivars [20], even in stress conditions such as water regimes [21]. Its positive effect on plant growth during salt stress in crops such as soybean *Glycine max* L. (Fabales: Fabaceae), [22] lettuce *Lactuca sativa* L., (Asterales: Asteraceae), and tomato seedlings *Solanum lycopersicum* L., (Solanales: Solanaceae) [23], allows plants to tolerate abiotic stress factors [24]. Its application can not only limit the use of agrochemicals [25] against grapevine root pathogens [26] but can enhance its metabolic and developmental characteristics [27], to cope with abiotic conditions. Salomon et al., 2014 [28] have reported that *Bacillus* strains act as stress alleviators in *V. vinifera* L., by inducing abscisic acid (ABA) synthesis and reducing water losses, while in a different study [29] described ABA production and accumulation in grapevine diminished plant water loss rate.

Sinorhizobium meliloti (Hyphomicrobiales: Rhizobiaceae), has been characterized as an endophyte for its mutualistic associations with many plants [30,31] as it migrates endophytically into most of the plant parts [32]. It is diazotrophic: it fixes the gaseous nitrogen into usable forms such as ammonia [33], so plant and *S. meliloti* can benefit nutritionally [34] and the use of nitrogen fertilizers can be reduced [35]. Many studies mention the symbiosis between *S. meliloti* and legumes [36] mostly for nitrogen fixation on roots. There is strong evidence of its acting-as-biostimulant effect on several plants such as fenugreek *Trigonella foenum-graecum* L., (Fabales: Fabaceae) under water deficit stress [37], on *L. sativa* [38], in black medic (*Medicago lupulina* L., (Fabales: Fabaceae) [39], on cowpea *Vigna unguiculata* L., (Fabales: Fabaceae) [40], and on *V. vinifera* [41] has been reported.

There is a research gap regarding the stimulatory potential that *S. meliloti* may present in grapevine cultivation under water stress conditions [42]. This research was aimed to evaluate the application of two commercial formulations, each of them containing *B. amyloliquefaciens* and *S. meliloti*, respectively, as biostimulants in grapevine cultivation under irrigation deficit conditions.

2. Materials and Methods

2.1. Experimental Design

In the experiment which lasted for 140 days, grapevine cuttings of the indigenous to Zitsa (Ioannina viticultural zone, Greece) Debina variety were used. The cuttings were placed under misting conditions, to acclimatize them and produce roots. The main goal of the experiment was to study the biostimulant effect of the commercial formulations as close to a realistic cultivation environment as possible. For this reason, clay soil from a vineyard in the area of the plain of Arta was used as a growth substrate. It is important to mention that the soil did not undergo any steam pasteurization treatment; therefore,

the data of this study could constitute the foreground for the future repetition of the experiment on the same field from where the soil was taken. Using this soil, an important first insight into the performance of the formulations was obtained in conditions as close as possible to grapevine growing reality. Also, this attempt is a first screening, on whether the preparations can compensate for the water deficit, based on laboratory indications concerning the metabolism of the vine culture. When the grapevine cuttings were planted in 9 L pots, initial granular fertilizer (N12-P12-K17) was applied.

In order to study the effect of commercial strains on grapevine metabolism under water deficit conditions, the experiment was carried out in the greenhouse of the Agriculture Department of the University of Ioannina in Kostakioi, Arta (Greece). A drip irrigation system was built for the experimental vine cultivation pots, which communicated with a central computer program (ARGOS Electronics 2014), and the pots were irrigated at regular intervals. The amount and frequency of irrigation were based on climate data from greenhouse temperature and relative moisture sensors. To assess the optimal irrigation needs of experimental grape cultivation in the greenhouse, monthly measurements of the evapotranspiration of some selected plants in the treatment pots were carried out. Plants from each experimental treatment were selected and checked monthly in order to calculate their water losses. Finally, two categories of irrigation were determined: optimal irrigation at 100% of available water (AW) and deficit irrigation at 57% of AW to achieve a balanced mid-level water deficit. Reaching 57% of the AW irrigation deficit level, on the one hand, the metabolism of the grapevine plants was stressed, and on the other hand, their viability under extreme conditions was ensured. In addition, the relative soil moisture was regularly checked with a soil moisture meter (ΔT-SM150 Kit, Delta-T Devices, Ltd., Cambridge, UK) to have a complementary picture of the irrigation adequacy of each treatment.

The experimental setup was organized in irrigation channels on the greenhouse benches, including 6 treatments in a completely randomized design. Each treatment had 3 replications and each replication consisted of 9 plants. The preparations were applied in conditions of rational irrigation 100% of AW, and deficit irrigation (57% of AW). Any treatment beginning with the letter K means that deficit irrigation (57% of AW) was applied. For this purpose, two control treatments were used: C (100% of AW) and KC (57% of AW). Two commercial bacterial-based formulations were used: the biostimulant HYDROMAAT, FUTURECO BIOSCIENCE® containing *Sinorhizobium meliloti* cepa B2352 (2% *w*/*w*) applied on SM (100% of AW) and KSM (57% of AW) treatments, and the biofungicide SERENADE ASO, BAYER® containing *B. amyloliquefaciens* QST 713 (1.34% *w*/*w*) applied on BA (100% of AW) and KBA (57% of AW) treatments.

The reasoning behind choosing to study these liquid formed formulations lies in their availability on the market. Moreover, they are recommended for a wide range of crops including grapevine. Lastly, the lack of any reported cases in the literature concerning the grapevine makes this choice appropriate. HYDROMAAT is an oil dispersion (OD), which under water stress conditions, can regulate the genetic expression of the BetS protein, a glycine-betaine transporter, a molecule involved in the cell's osmoregulation. It also regulates the proline dehydrogenase gene that plays a key role in the plant's response to drought through osmoregulation. SERENADE ASO is a suspension concentrate (SC), recommended for a wide range of crops as well as for the vine as a biological fungicide to combat diseases caused by *Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia* spp., and *Verticillium* spp. In addition, it has been reported for its biostimulant effect on buckwheat plants *Fagopyrum esculentum* [42].

2.2. Plant Growth Parameters

Plant growth parameters were estimated on each treatment. At the end of the experiment the total plant length (cm) (central shoot and lateral shoots) was measured with a portable meter and leaf area (cm^2) was estimated by the Image J protocol [43], by cutting the leaves of every plant on each treatment. Total plant biomass was ascertained by determining dry weight (g). Each plant on each treatment was separated into leaves, shoots, and

roots, cleaned with diH_2O, and after 48 h on 80 ± 1 °C, weighed on a precision electronic scale (KERN EG-N).

2.3. Leaf Relative Water Content

To ascertain plant stress the relative water content of the vine leaves was estimated. This method can give an easy and quick answer for the state of abiotic stress in plant leaves. According to the protocol of Bertamini et al., 2006 [44] discs were cut from grapevine leaf segments (0.3 g fresh mass) and weighed immediately to record their fresh mass (FW), on a precision balance and immediately immersed in diH_2O where they remained for 6 h in the dark at 4 °C.

The samples were then reweighed to record their turgid fresh mass (TW) and after 24 h at 80 ± 1 °C reweighed to obtain their dry mass (DW). The leaf relative water content (LRWC) of grapevine was calculated with the equation:

$$\text{LRWC} = [(\text{FW} - \text{DW})/(\text{TW} - \text{DW})] \times 100\%$$

2.4. Total Chlorophyll Content of V. vinifera Leaves

The amount of total chlorophyll (Ca + Cb) gives an insight into the health status of the plants and was calculated in a non-destructive way with the SPAD-502 device (Minolta Co., Ltd., Osaka, Japan). In order to test the accuracy of the measurements, the SPAD readings were positively correlated (R^2 = 0.9265) with the destructive chemical method of chlorophyll determination, according to the protocol of Uddling et al., 2007 [45]. As an extraction solvent, 10 mL of 100% acetone was used for 0.04 g of homogenized fresh leaf tissue (2.66 cm^2 leaf disc area). The grapevine leaf samples were crushed in a mortar with a pestle, poured into glass tubes, vortexed, and left overnight in the dark at 2–4 °C. The absorption of the samples was performed in a spectrophotometer (Jasco-V630 UV-VIS, Jasco International Co., Ltd., Tokyo, Japan) at 644.8 and 661.6 nm. The total chlorophyll content was calculated by the equations of Lichtenthaler and Buschmann, 2001 [46] and expressed in μg of fresh leaf per cm^2 of leaf area:

$$\text{Ca } (\mu\text{g/mL}) = 11.24 \times A_{661.6} - 2.04 \times A_{644.8}$$

$$\text{Cb } (\mu\text{g/mL}) = 20.13 \times A_{644.8} - 4.19 \times A_{661.6}$$

2.5. Determination of Proline

Grapevine leaves were sampled for the determination of proline at days 0, 56 and 122 of the experiment. The chemical determination of proline was performed according to the protocol of Carillo and Gibon, 2011 [47] with some modifications: 4 mL of 70% ethanol was used for 0.1 g of fresh homogenized grapevine leaf tissue and then centrifuged for 10 min at 4000× *g*. A total of 1 mL of the supernatant extracted solution and 2 mL of freshly prepared acid–ninhydrin solution were placed in a new test tube, and the final mixture was vortexed and incubated for 25 min in a water bath (95 °C). The reaction mixture was cooled in an ice bath until room temperature was achieved and then centrifuged for 5 min at 4000× *g*. The absorbance was determined in a spectrophotometer (Jasco-V630 UV-VIS, Jasco International Co., Ltd., Tokyo, Japan) at 520 nm. A calibration curve was established (R^2 = 0.9966), using proline solutions ranging from 0.025 to 0.8 mM, in the same medium as the one used for the extraction, and the data were reported in μmol of proline g^{-1} of fresh leaf weight.

2.6. Assessment of Total Phenolic Content

The determination of Total Phenolic Components (TPC) was carried out at the days 0, 56, and 122 of the experiment, by the method described by Katalinic et al., 2013 [48] with modifications. 0.1 g of dry leaf tissue was extracted with 80% ethanol solvent and centrifuged for 15 min in 3000× *g* at 12 °C. 250 μL of the supernatant extracted solution were diluted in a final volume of 10 mL diH_2O. In a new test tube, 1mL of the diluted

extract solution, 4.5 mL diH_2O, 500 µL of Folin–Ciocalteu 2N reagent, and after 3 min 4 mL of dehydrated Na_2CO_3 solution 7.5% w/v were added. The final mixture was vortexed and then incubated in a water bath (40 °C) for 20 min in the dark (room temperature). The absorption was recorded spectrophotometrically at 765 nm (Jasco-V630 UV-VIS) against the prepared blank. Gallic acid was used as a standard for the quantification of TPC and the results were reported in mg GAE (Gallic Acid Equivalent) g^{-1} of dry leaf weight.

2.7. Statistical Analysis

Statistical analysis of the mean values of the parametric data was made to evaluate the results by using the software SPSS v.26 (IBM-SPSS Statistics, Armonk, NY, USA). One-way ANOVA variance analysis was performed, and the Bonferroni criterion was utilized for statistical significance to compare the means, with a significance level of 5% ($p \leq 0.05$). Different letters between treatments indicate significant differences in each table according to the Bonferroni test.

3. Results

3.1. Plant Growth Parameters

In the present study, we observed that even in non-stress conditions (100% of AW), in the treatments where the formulations with *B. amyloliquefaciens* (BA) and *S. meliloti* (SM) were applied, a much greater total length of vegetation was noted compared to the control (Figure 1) (C = 183.89 ± 1.93 cm), with statistically significant difference. At the end of the experiment, the greatest promotion in *V. vinifera* total vegetation was observed in the BA (271.44 ± 3.34 cm) treatment, presenting a statistically significant difference with all the other treatments (F = 201.553, df = 5, $p < 0.001$) as shown on Table 1. The difference between KBA (238.0 ± 1.58 cm) and KC (147.0 ± 1.58 cm) was spectacular, presenting a statistically significant difference (F = 201.553, df = 5, $p < 0.001$).

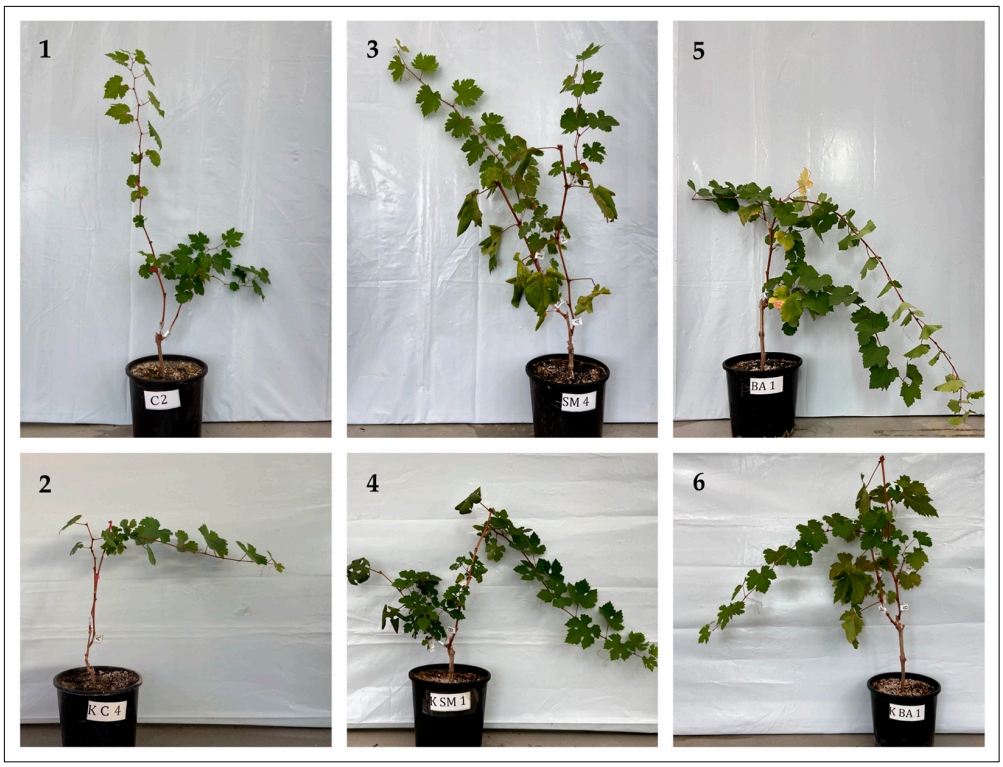

Figure 1. Representative plants of each treatment at the end of the experiment (Day 140): (**1**) C: 100% of AW, (**2**) KC: 57% of AW, (**3**) SM: 100% of AW + HYDROMAAT, (**4**) KSM: 57% of AW + HYDROMAAT, (**5**) BA: 100% of AW + SERENADE ASO, (**6**) KBA: 57% of AW + SERENADE ASO.

Table 1. Total length (cm) of shoots and leaf area (cm^2) of *V. vinifera* plants at the end of the experiment (Day 140). Different letters between treatments' mean values (±SE) indicate significant differences according to the Bonferroni test ($p \leq 0.05$).

Treatment	Total Shoots Length (cm)	Leaf Area (cm^2)
C (100%)	183.89 ± 1.93 d	1707.60 ± 207.36 c
KC (57%)	147.00 ± 1.58 e	1252.17 ± 85.0 dc
SM (100%)	237.22 ± 5.45 b	3449.37 ± 89.44 a
KSM (57%)	200.00 ± 3.16 c	2358.70 ± 51.11 bc
BA (100%)	271.44 ± 3.34 a	3382.10 ± 110.5 a
KBA (57%)	238.00 ± 1.58 b	2628.73 ± 150.41 b

An increased leaf area of grapevine plants was observed in the treatments with PGPR both of stressed and non-stressed conditions (Table 1). In addition, both treatments with *S. meliloti* (SM = 3449.37 ± 89.44 cm^2) and *B. amyloliquefaciens* (BA = 3382.10 ± 110.5 cm^2) formulation, promoted the final leaf area, under optimal irrigated conditions with a statistically significant difference to the control (C = 1707.60 ± 207.36 cm^2) (F = 48.827, df = 5, $p < 0.001$). A proportionally positive picture was also recorded in the case of deficit irrigation (57% of AW) where the KBA (2628.73 ± 150.41 cm^2) treatment achieved the maximum increase in the leaf surface of the grapevine plants presenting a statistically significant difference with the deficit irrigated control (KC = 1252.17 ± 85.0 cm^2) (F = 48.827, df = 5, $p < 0.001$).

As shown on Table 2, in each treatment the root dry weight occupies the largest percentage of the total dry biomass of the grapevine plants, followed by the shoots and leaves dry weight. At the end of the experiment, the total plant dry biomass of the deficit irrigated control (KC = 28.5 ± 1.3 g) differs significantly from the optimal irrigated control (C = 52.7 ± 3.4 g) (F = 196.869, df = 5, $p = 0.002$), as it was quite difficult to adapt to such less available water. In contrast, grapevine deficit irrigation treatments to which PGPRs were applied, not only overcame this barrier (KSM = 103.3 ± 2.4 g, KBA = 97.4 ± 1.3 g), but managed to increase the total dry weight of roots, shoots, and leaves, annotating a statistically significant difference to the control (KC) (F = 196.869, df = 5, $p < 0.001$).

Table 2. Dry biomass of *V. vinifera* plants at the end of the experiment (Day 140). Different letters between treatments' mean values (±SE) indicate significant differences according to the Bonferroni test ($p \leq 0.05$).

Treatment	Roots Dry Weight (g)	Shoots Dry Weight (g)	Leaves Dry Weight (g)	Total Dry Biomass (g)
C (100%)	34.3 ± 5.1 c	11.1 ± 0.4 d	7.3 ± 2.0 cd	52.7 ± 3.4 c
KC (57%)	17.9 ± 2.5 c	6.7 ± 0.9 d	3.9 ± 0.7 d	28.5 ± 1.3 d
SM (100%)	83.5 ± 2.8 ab	26.3 ± 0.9 ab	22.7 ± 0.8 ab	132.4 ± 1.5 a
KSM (57%)	68.9 ± 3.3 b	19.0 ± 1.2 c	15.4 ± 0.3 abc	103.3 ± 2.4 b
BA (100%)	89.7 ± 4.2 a	28.3 ± 1.7 a	24.1 ± 3.5 a	142.1 ± 6.1 a
KBA (57%)	62.1 ± 1.4 b	19.4 ± 2.6 bc	13.2 ± 2.3 bcd	94.7 ± 1.3 b

3.2. Leaf Relative Water Content

Both SM (92.4 ± 0.5%) and BA (93.6 ± 0.3%) treatments on grapevine appeared to have a better performance on LRWC with a statistically significant difference compared to the control (C = 73.7 ± 0.7%) (F = 492.082, df = 5, $p < 0.001$). Additionally, both bacterial-based formulations helped grapevine plants to improve their LRWC even under stress conditions, giving them strength and stability compared to the deficit control (KC = 57.8 ± 1.1%) with a statistically significant difference (F = 492.082, df = 5, $p < 0.001$), as presented on Table 3, while *B. amyloliquefaciens* formulation KBA (90.2 ± 0.2%) proved to be better with a statistically significant difference than the formulation with *S. meliloti* KSM (85.1 ± 0.6%) (F = 492.082, df = 5, $p = 0.002$).

Table 3. Leaf relative water content (Day 69). Different letters between treatment's mean values (±SE) indicate significant differences according to the Bonferroni test ($p \leq 0.05$).

Treatments	LRWC (%)
C (100%)	73.7 ± 0.7 d
KC (57%)	57.8 ± 1.1 e
SM (100%)	92.4 ± 0.5 ab
KSM (57%)	85.1 ± 0.6 c
BA (100%)	93.6 ± 0.3 a
KBA (57%)	90.2 ± 0.2 b

3.3. Total Chlorophyll Content

During the experiment in control (C) the total chlorophyll content (TCHL) either slightly increases or remains relatively stable in *V. vinifera* leaves, while during water deficit (KC) the content drops even more, widening the difference between them. This difference is compensated by each of the PGPR formulations applied, as shown on Table 4. In the middle of the experiment, the BA treatment showed a higher TCHL content (39.75 ± 0.41 μg cm^{-2}) followed by SM (39.28 ± 0.38 μg cm^{-2}) with a statistically significant difference with C (30.60 ± 0.3 μg cm^{-2}) (F = 430.745, df = 5, $p < 0.001$). The PGPR formulations used in the experiment, keep the chlorophyll levels high even in the case of water deficit stress (KSM = 35.16 ± 0.19 μg cm^{-2}, KBA = 36.22 ± 0.25 μg cm^{-2}) with a statistically significant difference with KC (20.84 ± 0.45 μg cm^{-2}) (F = 430.745, df = 5, $p < 0.001$). This pattern continues until the end of the experiment (Day 140), with the PGPR formulations showing a much higher content of TCHL than the controls, with statistically significant differences either in conditions of optimal (SM = 38.97 ± 0.36 μg cm^{-2}, BA = 37.34 ± 0.27 μg cm^{-2}, C = 23.59 ± 0.48 μg cm^{-2}) (F = 609.757, df = 5, $p < 0.001$) or deficit irrigation (KSM = 35.01 ± 0.12 μg cm^{-2}, KBA = 33.88 ± 0.16 μg cm^{-2}, KC = 17.92 ± 0.46 μg cm^{-2}) (F = 609.757, df = 5, $p < 0.001$).

Table 4. Total chlorophyll Content (TCHL) in the leaves of young *V. vinifera* plants at the beginning, at midterm, and at the end of the experiment sampling (μg cm^{-2} Fresh Leaf). Different letters between treatments' mean values (±SE) indicate significant differences according to the Bonferroni test ($p \leq 0.05$).

Treatment	TCHL μg cm^{-2} (Day 0)	TCHL μg cm^{-2} (Day 74)	TCHL μg cm^{-2} (Day 140)
C (100%)	25.67 ± 0.31 ab	30.60 ± 0.3 d	23.59 ± 0.48 e
KC (57%)	26.60 ± 0.29 a	20.84 ± 0.45 e	17.92 ± 0.46 f
SM (100%)	25.67 ± 0.28 ab	39.28 ± 0.38 a	38.97 ± 0.36 a
KSM (57%)	26.14 ± 0.29 ab	35.16 ± 0.19 c	35.01 ± 0.12 c
BA (100%)	25.98 ± 0.30 ab	39.75 ± 0.41 a	37.34 ± 0.27 b
KBA (57%)	25.41 ± 0.51 b	36.22 ± 0.25 bc	33.88 ± 0.16 cd

3.4. Total Phenolic Content

PGPR inoculants can modulate plant antioxidant enzymes, enriching plants' metabolic capability by reducing ROS levels [49]. This picture agrees with the state of total phenolic components in all treatments with PGPR formulations, and this possibly constitutes an important indication of the experiment on the metabolic behavior of grapevine plants under deficit irrigation conditions. As presented in Table 5, on day 56 we observed that the deficit irrigation treatment with *S. meliloti* KSM (46.99 ± 1.17 mg GAE g^{-1}) accumulated more total phenolics than the deficit irrigation KC (33.69 ± 0.28 mg GAE g^{-1}) treatment with a statistically significant difference (F = 68.373, df = 5, $p < 0.001$), a pattern that continues into the day 122 (KSM = 28.92 ± 1.62 mg GAE g^{-1}, KC = 18.12 ± 0.27 mg GAE g^{-1}) (F = 45.927, df = 5, $p < 0.001$).

Table 5. Total Phenolic Content (TPC) in the leaves of young *V. vinifera* plants at the days 0, 56 and 122 of the experiment sampling (mg GAE g^{-1} Dry Leaf). Different letters between treatments' mean values (±SE) indicate significant differences according to the Bonferroni test ($p \leq 0.05$).

Treatment	TPC mg GAE g^{-1} (Day 0)	TPC mg GAE g^{-1} (Day 56)	TPC mg GAE g^{-1} (Day 122)
C (100%)	23.83 ± 0.77 a	27.67 ± 0.26 cd	12.30 ± 0.18 c
KC (57%)	19.78 ± 1.0 a	33.69 ± 0.28 bc	18.12 ± 0.27 b
SM (100%)	19.88 ± 1.26 a	31.51 ± 1.12 c	17.08 ± 1.28 bc
KSM (57%)	19.15 ± 0.72 a	46.99 ± 1.17 a	28.92 ± 1.62 a
BA (100%)	22.06 ± 1.10 a	32.86 ± 1.0 c	14.59 ± 1.05 bc
KBA (57%)	22.17 ± 1.02 a	37.85 ± 1.18 b	26.22 ± 1.45 a

3.5. Proline Content

The amount of proline on *V. vinifera* leaf samples from the beginning of the experiment (Day 0) is at relatively optimal levels in all of the treatments (range between 0.103 and 0.112 ± 0.003 μmol g^{-1}) without statistical differences between them, while during the irrigation stress (57% of AW) at day 56 it remains low in the water deficit control (KC = 0.105 ± 0.003 μmol g^{-1}) compared to the treatments where PGPR formulations were applied with a statistically significant difference with KBA (0.171 μmol g^{-1}) (F = 201.400, df = 5, $p < 0.001$) and with KSM (0.168 ± 0.003 μmol g^{-1}) (F = 201.400, df = 5, $p < 0.001$). Then, close to the end of the experiment at day 122, the optimal irrigated control C (0.080 μmol g^{-1}) and the deficiently irrigated control KC (0.097 μmol g^{-1}) remained in low levels of proline, while in the stressed KSM (0.160 ± 0.003 μmol g^{-1}) and KBA (0.185 ± 0.003 μmol g^{-1}) treatments was observed increased proline accumulation in leaf plants' tissues, in response to abiotic conditions, with a statistically significant difference to the KC (F = 321.133, df = 5, $p < 0.001$), as shown in Table 6.

Table 6. Proline content in the leaves of young *V. vinifera* plants at the days 0, 56 and 122 of the experiment sampling (μmol g^{-1} Fresh Leaf). Different letters between treatments' mean values (±SE) indicate significant differences according to the Bonferroni test ($p \leq 0.05$).

Treatment	Proline μmol g^{-1} (Day 0)	Proline μmol g^{-1} (Day 56)	Proline μmol g^{-1} (Day 122)
C (100%)	0.104 ± 0.003 a	0.068 ± 0.0 d	0.080 ± 0.0 d
KC (57%)	0.109 ± 0.003 a	0.105 ± 0.003 c	0.097 ± 0.0 c
SM (100%)	0.111 ± 0.0 a	0.129 ± 0.003 b	0.090 ± 0.0 cd
KSM (57%)	0.115 ± 0.003 a	0.168 ± 0.003 a	0.160 ± 0.003 b
BA (100%)	0.103 ± 0.003 a	0.106 ± 0.003 c	0.096 ± 0.003 c
KBA (57%)	0.112 ± 0.0 a	0.171 ± 0.0 a	0.185 ± 0.003 a

4. Discussion

In the present study, the applications of formulations containing PGPR showed a stimulating effect on grapevine saplings *V. vinifera* in pot conditions, under deficit irrigation 57% of AW. Plant growth parameters such as length showed a significant quantitative increase in the application of PGPR formulations than in their absence, compared to the controls, and this result agrees with other works in which PGPR was applied [50,51].

Our results in PGPR treatments on increased roots and shoots dry biomass agree with those of Asari et al., 2017 [52], where *B. amyloliquefaciens* inhibited primary root growth on *Arabidopsis thaliana* (Brassicales: Brassicaceae), an event that may be due to increased cytokinin levels on roots or because of the increased auxin levels that were detected on colonized roots. Also, our results agree with Vardharajua et al., 2010 [53], where five *B. amyloliquefaciens* strains inoculated on maize *Zea mays* (L.) (Poales: Poaceae) increased root and shoot dry biomass under stress and non-stress conditions. For *S. meliloti*, although it has been studied on a smaller scale, studies show that it creates an activating environment

in the root of grapevine plants [41,54] including volatile organic compounds (VOCs) that are involved in stress defense mechanisms and play an important role in grapevines interaction with the environment.

An increased leaf surface of grapevine plants was observed in the stressed and non-stressed treatments with PGPR and this fact, according to the study by Horák et al., 2021 [55], in combination with the increased weight of the leaves, could be indicative of an improvement in grape must quality. The increase in leaf area by the rhizobacterium *B. amyloliquefaciens* has been mentioned before [51] and agrees with the results of the present research.

Biological plant protection preparations are not related to plant growth promotion. However, when it comes to formulations containing PGPR, phenomena that benefit plant growth can occasionally be observed, if these formulations are applied with specific repeatability and dosage. PGPR contained in commercial formulations may enhance crops' resistance to harsh environmental conditions such as drought stress [56–58]. In the study of Witkowicz et al., 2019 [42] the *B. amyloliquefaciens*-based commercial formulation Serenade Aso was tested for its biostimulant potential on growth and nutrition of *F. esculentum* (Caryophyllales: Polygonaceae), and resulted in a better-quality sprout production, a result that is in agreement with the beneficial effect of the same formulation in our findings, although the high levels of dry matter that was observed in the results of our work, is not reported in their research.

We also observed a high performance of PGPR formulations on plant stress indicators such as on leaf relative water content which agrees with the results of He et al., 2021 [59] where the *Bacillus* spp., was inoculated on ryegrass *Lolium perenae* seed (Poales: Poaceae), improving leaf relative water content. PGPR inoculants can modulate plant antioxidant content, enriching plant metabolism by reducing ROS levels [49].

Total phenolic components are products of the plant's secondary metabolism and are an indicator of the metabolic status of grapevine plants [60]. Phenols and polyphenols such as flavonoids act as antioxidants against cytotoxic toxic effects of oxygen radicals. The content of total phenolic components in all *V. vinifera* treatments with PGPR was significantly increased compared to the controls, especially in the case of treatments where water stress was implemented, a fact that may be an important indicator of the behavior of grapevine plants in deficit irrigation conditions. The higher content of total phenolics in the deficit irrigation treatment with PGPR containing *S. meliloti* may be an indication of its promoting effect in abiotic stress environments, as the accumulation of phenolics enhances plant metabolism, improving antioxidant enzyme production [61] to cope with the abiotic stress, which is supported by the results of studies by Bianco & Defez 2009 [62] with the application of *S. meliloti* in barrelclover *Medicago truncatula* (Fabales: Fabaceae) under salt stress. In our results, a suchlike status on the phenolic content appeared on the irrigation-stressed *V. vinifera* treatments to which they were applied *S. meliloti* (KSM) formulation, a depiction that has presented in other grapevine experiments, where an increased content of TPC is observed as a result of the application of PGPR [63].

Proline shows a similar picture to the total phenolic components in our work. Proline accumulation is a known metabolic response when higher plants are exposed to water stress conditions and acts as an osmolyte in drought conditions and is involved in osmoregulation [64]. Moreover, several studies have suggested a ROS scavenger role so its presence prevents plant oxidative damage [49,50,65,66]. Both commercial formulations were able to protect grapevine plants from water stress by increasing the content of proline in plant tissues, a fact that also applies to the work of Theoharis et al., 2012 [67] where the addition of PGPR enhanced the accumulation of proline in grapevine tissues under cold stress. The same effect is described by Tiwari et al., 2017 [68] where *B. amyloliquefaciens* application increased proline levels in a rice crop under saline stress, and by Bittencourt et al., 2023 [49] and Vardharajula et al., 2010 [53] in maize crops under drought stress. In our results, contrary to the treatments that did not applied PGPR formulations, ROS did not undergo the same neutralization, due to the comparatively much lower accumulation of proline in

the plant tissues, so we can assume that the oxidative damage was greater. This result is in agreement with the result of the rest stress indicators, such as the LRWC and total phenolic content, which were very low in these treatments. The rhizobacteria *B. amyloliquefaciens* and *S. meliloti* contained in the commercial formulations seem to enhance plant metabolism, increasing the accumulation of proline, thus creating a promoting environment that helps plants to recover more rapidly from the oxidative damage, based on the picture observed in other works [69,70].

The content of total chlorophyll either increases slightly or remains relatively constant in grapevine plants, while in the case of water stress the content decreases more, widening the difference between them. This difference is not only prevented by each of the PGPR formulations applied, but was overcompensated, boosting the plant metabolic defense mechanisms. Firstly, this difference may be because *B. amyloliquefaciens* and *S. meliloti* contribute to biological nitrogen fixation [71]; secondly, the *B. amyloliquefaciens* regulation of cytokinins [65] plays a role in the physiological processes such as chlorophyll accumulation [52,72]. This picture of increased chlorophyll content in the BA and SM treatments was also presented in our previous work [73] on grapevine saplings *V. vinifera*.

Since there are no available data in the literature, the purpose of this computation was to underline the magnitude of water compensation that could be achieved by the application of PGPR *B. amyloliquefaciens* and *S. meliloti* formulations in the greenhouses, for the production of vine cuttings. In addition, this work may demonstrate a potential for the experimental research of the specific PGPR formulations at the level of young plants in the field, due to the encouraging results in the developmental and metabolic characteristics, which can find applications in semi-drought field conditions, helping to compensate for the lack of water. A considerable parameter of the existence of PGPR in a greenhouse environment is the elevated temperature that may slow down their population, as many beneficial bacteria thrive under lower temperatures [74]. On the other hand, high temperature for PGPRs like *Bacillus* spp. does not seem to be a deterrent factor, as several works present their successful action under intense elevated temperatures of greenhouses [75–77], although optimal temperature conditions are a parameter that could be controlled in nurseries, for the establishment of new vineyards.

5. Conclusions

Drought, especially under elevated temperatures, can cause vegetative and biochemical changes in many Mediterranean grape varieties, and for this reason, it is important to approach alternative perspectives, regarding the management of grapevine cultivation, given climate change. In our study, these adverse conditions were simulated in the greenhouse, and our research showed that the two rhizobacteria commercial formulations managed to overcome the abiotic obstacles caused by deficit irrigation and benefit the metabolism and growth of grapevine plants. In the treatments where PGPR formulation were applied the growth of the grapevine plants was more improved, compared to the controls where no formulations were applied, as this was shown by the growth parameters such as the total length, leaf area, and total fresh and dry weight. The PGPR formulations additionally improved the total phenolic content and proline levels of grapevine, even under water deficit conditions.

This research initiates the discussion for upcoming studies for *S. meliloti* and corresponding PGPR formulations on grapevine, as it has been studied mostly on legumes. Additionally, we tried to simulate field soil conditions in greenhouse pots, choosing to place the vineyard soil intact under normal and deficit irrigation conditions. More field studies need to verify these results in real grape growing conditions and even more important is to investigate, if there is an impact on grape production, as some PGPR formulations enhance to a certain degree, the grape production. A future perspective in the experimentation with these formulations would be the observation of possible interactions between the microorganisms present in the soil and the microorganisms contained in PGPR formulations. For this reason, the microbiological analysis of the soil would provide valuable insights, so

that there is a more accurate depiction of the mode of action of those PGPR formulations. Finally, we consider important for the future to test the formulations in steam-pasteurized soil, to obtain data on the specific mode of action of the microorganisms contained in these formulations, for the cultivation of the grapevine.

Nevertheless, the behavior investigation of grapevine plants treated with beneficial bacterial formulations, under water scarcity on field experiments, may give valuable insights in plants' resistance. Regarding our results, we consider it necessary for a more vigorous verification of the biostimulant action of some commercial formulations containing PGPR and exist on the market as biological insecticides.

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