Environment Protection Engineering

Vol. 51 2025 No. 1

DOI:10.37190/epe250107

LAMIA BICHARA[1, 2], AIMAN SLIMANI [2, 3], ANAS RAKLAMI[2]
TARIK SAHLAOUI[2], ABDEL-ILAH TAHIRI[2], KHADIJATTOU TAOUFIQ[1]
KHOUZAIMA EL BIARI[4], MUSTAPHA FAGHIRE1[1]
KHALID OUFDOU (ORCID: 0000-0001-7201-2041)[2, 5]

PLANT GROWTH-PROMOTING RHIZOBACTERIA ISOLATED FROM ARGAN TREE SOIL INDUCING SYSTEMIC DROUGHT TOLERANCE IN ALFALFA

The occurrence of climate change has resulted in extremely dry climatic conditions, which led to a significant impact on crop growth and productivity. The rhizosphere of the endemic argan forest could be a baring ground for naturally unexplored plant growth-promoting rhizobacteria (PGPR). A greenhouse experiment was conducted to evaluate the effect on alfalfa plants under drought conditions of four selected rhizobacteria, including two actinobacteria, in combination with two naturally endosymbiont *Ensifer meliloti* strains (RhOL). A completely randomized design was used with water stress as the main factor consisting of two regimes: (1) well-watered plants (75% field capacity (FC)) and (2) water-stressed plants (35% FC), and PGPR inoculation as a second factor consisting of four inoculation treatments: control, consortia (LBA8 + LBA19 + RhOL6 + RhOL8 strains), (3) consortia 2 (LB4 + LBP2 + RhOL6 + RhOL8 strains), and (4) consortia 3 (LBA8 + LBA19 + LB4 + LBP2 + RhOL6 + RhOL8 strains). Inoculated alfalfa plants showed tolerance to drought stress by increased production of total chlorophyll and osmolytes, including proteins and sugars, under drought stress. The bacterial inoculation led to a lower H_2O_2 content in alfalfa leaves and neutralized the reactive oxygen species. These PGPR strains appeared to be important tools capable of being developed into bioinoculants to effectively improve drought tolerance in plants in a sustainable agriculture strategy.

[1]Laboratory of Plant Biotechnology, Department of Biology, Faculty of Sciences, Ibn Zohr University (UIZ), Agadir, Morocco.

[2] Cadi Ayyad University, Faculty of Sciences, Semlalia, Laboratory of Water Sciences, Microbial Biotechnologies, and Natural Resources Sustainability (AQUABIOTECH), Unit of Microbial Biotechnologies, Agrosciences and Environment (BIOMAGE)-CNRST Labeled Research Unit No. 4, Marrakech, 40000, Morocco, corresponding author K. Oufdou, email address: oufdou@uca.ac.ma

[3]Laboratory of Agro-Food, Biotechnologies and Valorization of Plant Bioresources (Agrobioval), Faculty of Sciences Semlalia, Cadi Ayyad University, Marrakesh, Morocco.

[4]Laboratory of Biology, Ecology and Health, Faculty of Sciences, Abdelmalek Essaadi University, Tetouan, Morocco.

[5]AgroBiosciences program, College for Sustainable Agriculture and Environmental Sciences, University Mohammed VI Polytechnic (UM6P), Benguerir, Morocco.

1. INTRODUCTION

Due to climate change, drought poses a critical challenge, imposing a significant impact on crop growth and productivity worldwide [1]. The consequences of a water deficit on plants are profound, threatening key physiological processes vital for their survival and affecting nutrient availability [2]. Drought also leads to the overproduction and accumulation of reactive oxygen species (ROS) arising from oxygen metabolism, which inflicts oxidative damage upon essential cellular components, including proteins, DNA, lipids, and even cells themselves [3]. Therefore, it is necessary to find a strategy for improving crop tolerance against water scarcity.

In recent years, researchers have focused their attention on Plant Growth-Promoting Rhizobacteria (PGPR) as a promising solution to mitigate the adverse effects of drought on plants [4]. These beneficial bacteria, residing in the rhizosphere, exhibit diverse mechanisms that enhance plant growth and development [4]. One of the key mechanisms through which PGPR aids plants in combating water scarcity is by facilitating nutrient uptake [5]. They can solubilize phosphate and fix atmospheric nitrogen, making these essential nutrients more available to plants even under limited water availability. Furthermore, PGPR can produce plant growth-promoting substances such as phytohormones, including auxin, cytokinin, and gibberellin [6]. These phytohormones regulate various physiological processes in plants, such as root development, water uptake, and stomatal conductance, ultimately leading to improved water-use efficiency and reduced transpiration rates during water-stressed conditions [7]. PGPR can also trigger the synthesis of osmoprotectants, playing a role in the management of osmotic stress caused by water deficiency [4]. By accumulating these osmoprotectants, plants can better maintain cellular water balance and mitigate the detrimental effects of dehydration [8]. Another crucial way in which PGPRs assist plants in resisting water stress is through the modulation of antioxidant enzymes [4]. They enhance the activity and expression of key antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) in plants, which help plants maintain a balanced redox state and protect cellular structures and molecules from oxidative damage induced by water stress [3].

The argan tree *Argania spinosa* (L.) skeels, the only representative of the *Argania* genus is a distinctive evergreen endemic to the arid and semi-arid zones of southwest Morocco [9]. It plays an important role in terms of its botanical, ecological, and economic interests as well as its social value [10]. The argan tree preserves the ecosystem and provides a favorable environment for maintaining floral and faunal biodiversity. Furthermore, the natural argan forest faces major environmental challenges due to its geographical location on the border of one of the world's hottest deserts, characterized by highly arid climatic conditions and strongly threatened by the impacts of global climate change [10].

In this context, our study was designed to investigate the beneficial effect of bacterial consortia composed of indigenous strains isolated from rhizospheric soil of argan

trees of Agadir region, in improving drought stress tolerance in alfalfa plants. They were selected based on their abiotic stress tolerance and their PGPR activities. Instead of a single strain inoculation, the employment of a combination of microorganisms such as actinobacteria, N_2-fixing rhizobia, and other rhizosphere bacteria on alfalfa subjected to drought stress has received limited research attention and may contribute to the development of innovative and environmentally friendly strategies to address the challenges posed by the climate change. According to our knowledge, no study used both these consortia on alfalfa plants and mitigating drought stress.

2. MATERIALS AND METHODS

Study site and soil sample collection. Soil samples were collected, in March 2021, from five distinct locations in the Sous-Massa region, with a particular focus on the argan tree (*Argania spinosa* (L.)) rhizosphere, a significant and well-known tree species native to Morocco, particularly in the semi-arid regions of southwestern Morocco [10]. These sites are located at different elevations ranging from 60 to 150 m above sea level and are characterized by an annual average temperature of around 20–25 °C, while the average annual rainfall is less than 250 mm [11]. Before sample collection, the shallow soil layer (0–10 cm) was carefully removed to guarantee an appropriate representation of the rhizosphere microbial community. Targeting this area (argan tree rhizosphere) was crucial since it is known to contain a diverse array of microorganisms with possible ecological significance [12]. The collected rhizosphere soil samples were promptly transferred into sterile plastic bags and immediately stored at 4 °C until subsequent bacterial isolation.

Phosphate solubilizing bacteria (PSB) isolation. Bacterial isolation was done according to the method described by Slimani et al. [4]. 10 g of each soil were agitated for 30 min in 90 cm^3 of sterile physiological water. Then, each sample was diluted and 100 µL was spread on Petri dishes (in tri-replicate) containing the National Botanical Research Institute's Phosphate growth medium without yeast extract (NBRIP) [13], which contained 5 g/dm^3 of $Ca_3(PO_4)_2$ as the only inorganic phosphate source. The plates were incubated at 28 °C for up to 15 days. Strains surrounded by a halo around the colony were selected for further analysis, as the halo indicated the solubilization of tricalcium phosphate by the production of organic acids, demonstrating the strain's ability to access and utilize this inorganic phosphate source [13]. The selected strains were then purified by repeated streaking on Bennett medium, containing 50 $\mu g/cm^3$ of cycloheximide to inhibit the growth of fungi and 10 $\mu g/cm^3$ of nalidixic acid to inhibit the growth of gram-negative bacteria, for actinobacteria, or on TSA (trypticase soy agar) plates for the other bacteria [14]. Pure strains were obtained by the quadrant method and stored on their respective medium at 4 °C until subsequent analysis. The strains were identified based on the 16S DNA sequencing at Macrogen, Inc. (Seoul, South Korea),

as *Halalkalibacterium* sp. for LB4 strain, *Bacillus* sp. for LBP2 strain, and *Streptomyces* sp. for LBA8 and LBA19 strains.

Drought and salt stress tolerance. The isolated bacterial strains obtained from the rhizosphere of native argan trees were tested for their ability to tolerate drought stress as described by Raklami et al. [15]. To assess their response to drought conditions, we utilized PEG6000 (polyethylene glycol M = 6000 g/mol) and NaCl as drought and salt-inducing substances, a well-established method for simulating water stress in laboratory experiments [14]. In a microtiter plate, the bacterial strains were subjected to increasing concentrations of PEG 6000 (representing different levels of drought stress) in the range of 40–0.75% in triplicates. The same PSB were also separately incubated in salt concentrations ranging from 2 M to 62 mM. The microtiter plate was incubated at 28 °C and the absorbance at 600 nm was monitored each day for 5 days using a microtiter plate reader (800 TS Absorbance Reader, BioTek, Winooski, USA).

In vitro screening of plant growth-promoting properties. The capacity of the isolated bacteria to promote plant growth was investigated through their ability to solubilize phosphate and potassium, IAA (indole acetic acid), siderophores, HCN production, and N_2 fixation. To assess their solubilization capacity, the NBRIP liquid medium containing tricalcium phosphate as the sole source of phosphorus was used. The experimental setup involved washing bacteria once with physiological water after their cultivation in their respective media. Then, Erlenmeyer flasks containing 100 cm^3 of NBRIP were inoculated with 0.2 cm^3 of bacterial strains after adjusting their absorbance at 600 nm to a standardized value of 0.1 (10^8 CFU/cm^3). Notably, for actinobacteria, direct inoculation was performed (due to the difficulties in adjusting their absorbance) [14]. Following a four-day incubation period at 28 °C and 140 rpm, samples (15 cm^3) were collected from the Erlenmeyer flasks by centrifugation at 6000 rpm for 10 min, and the solubilized phosphate was quantified using a colorimetric method based on the Olsen and Sommers technique [16]. To evaluate potassium (essential macronutrient for plant growth) solubilization, the Alexandrov agar medium was used: 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCO_3$, 0.006 g of $FeCl_3$, 2 g of KH_2PO_4, 20 g of agar, and 5 g of mica as an insoluble source of potassium. The ability was tested using the drop-plate method as described by Bechtaoui et al. [17]. The plates were incubated at 28 °C for 15 days. Results are expressed as the ratio halo diameter/colony diameter (HD/CD) at 7 days and 15 days after incubation. To quantify IAA production, the strains were inoculated, in three replicates of each strain, in Erlenmeyer flasks containing 100 cm^3 of Luria–Bertani medium supplemented with 1.02 g of L-tryptophan, a precursor required for IAA synthesis and incubated at 28 °C for 4 days for the bacteria, and 7 days for the actinomycetes, with continuous agitation (140 rpm). Following incubation samples were taken and the supernatant was obtained after centrifugation at 6000 rpm for 10 min. To quantify the IAA content in the supernatants, a colorimetric assay based on the Salkowski

reagent was utilized. Specifically, 1 cm^3 of the supernatant was mixed with 2 cm^3 of Salkowski reagent and incubated in the dark for 30 min. The optical density of the resulting mixture was then measured at 530 nm, and the content was calculated according to a standard curve. To estimate siderophore production, strains were inoculated into Chrome Azurol S (CAS) agar plates and then incubated at 30 °C for 15 days [18]. The transition from blue to orange/yellow around the colony indicated that a particular strain could generate siderophores and is considered a positive result. The diameter of the orange halo zone was measured in 7 and 15 days within 3 replicates of each strain. Hydrogen cyanide production (HCN) was assessed following the method described by Lorck [19]. Bacterial strains were streaked on a nutrient agar medium supplemented with glycine (4.4 g/dm^3). On the top lids of the Petri plates, Whatman filter paper saturated with an alkaline picric acid solution (2% Na_2CO_3 in 0.5% picric acid) was placed. The Petri plates were then sealed with parafilm and incubated at 30 °C for 4 days. A positive result was indicated by a color change of the Whatman paper from yellow (resulting from the sodium picrate solution) to orange or brown, indicating the production of HCN by the tested isolate. The capacity of the strains to fix atmospheric nitrogen was evaluated using a repeated streaking technique on N-free Jensen's media. After 3 days of incubation at 28 °C, the strains that demonstrated successful growth in this nitrogen-depleted medium without any external nitrogen source were identified as free nitrogen-fixing isolates [20].

Selection of bacterial consortia and in vitro strains computability test. Through a rigorous screening and evaluation screening process, four bacterial strains: *Halalkalibacterium* sp. (LB4), *Bacillus* sp. (LBP2), and *Streptomyces* sp. (LBA8 and LBA19) were selected based on their ability to withstand abiotic stress (drought and salt stress) and promote plant growth, following the earlier mentioned *in-vitro* tests. Additionally, two rhizobia strains (RhOL6 and RhOL8) identified as *Ensifer meliloti*, able to form a symbiosis with alfalfa, form nodules and biologically fix nitrogen, from our laboratory collection were carefully chosen for their beneficial ability to promote seed germination and plant growth under different conditions [6]. Subsequently, these selected bacterial strains were combined to form three distinct consortia, each designed to maximize their synergistic effects on plant growth. consortium 1 comprised *Streptomyces* sp. LBA8 strain, *Streptomyces* sp. LBA19 strain, *Ensifer meliloti* RhOL6 strain, and *Ensifer meliloti* RhOL8 strain, while consortium 2 consisted of *Halalkalibacterium* sp. LB4 strain, *Bacillus* sp. LBP2 strain, *Ensifer meliloti* RhOL6 strain, and *Ensifer meliloti* RhOL8 strain. Lastly, consortium 3 was an inclusive combination of all the chosen strains: *Streptomyces* sp. LBA8, *Streptomyces* sp. LBA19, *Halalkalibacterium* sp. LB4, *Bacillus* sp. LBP2, *Ensifer meliloti* RhOL6, and *Ensifer meliloti* RhOL8. The establishment of these consortia was purposefully designed to consider the distinctive qualities of each bacterial strain to create potent symbiotic connections that improve plant productivity and

health. These consortia were tested for their compatibility in solid medium. No inhibition was observed for all the consortia made.

Plant materials, experimental design, treatments, and growth conditions. A greenhouse experiment was carried out to evaluate the potential of the selected bacterial consortia in mitigating drought stress in Medicago sativa, as a plant model. To begin the experiment, the seeds of the *M. sativa* Demnate variety (landrace variety) were meticulously disinfected using sodium hypochlorite diluted to 1/5 for 5 min then rinsed 5 times with sterile distilled water. Subsequently, the disinfected seeds were soaked for 24 h in sterile distilled water and allowed to germinate. Once the germination process was complete, the germinated seeds were inoculated by soaking them for 30 min in 10 cm^3 of the selected consortia at a concentration of 10^9 CFU/cm^3 (equivalent to 1 at the optical density of 600 nm). A control was conducted under the same conditions using distilled water. The seedlings were carefully transferred into plastic pots filled with 2 kg of agricultural soil at the rate of 10 seeds/pot. An inoculation booster was scheduled by applying 2 cm^3 of the respective treatment one, two, and three weeks after planting.

The experiment design involves studying the effect of two factors (factorial experimental design). The first factor was drought stress, at two levels (well-watered (WW) and water stress (WS)), and the second factor was bacterial inoculation, at four levels (control without inoculation (Control), inoculation with consortium 1 (T1), inoculation with consortium 2 (T2), inoculation with consortium 3 (T3)). To simulate drought conditions, two different levels of water availability were applied: 75% field capacity (well-watered control) and 35% field capacity (drought-stress treatment).

The plants were grown for two months in the following daylight conditions under the greenhouse: daylight photoperiods of 250–1000 μmol/(m^2·s), average temperatures of 25/21 °C day/night, and relative humidity of 40% to 60%.

Harvest and plant growth measurement. After two months of cultivation, the plants were carefully uprooted, and the shoot was separated from the roots. The roots were then gently washed to remove any adhering soil particles. Subsequently, the length of both the shoot and the roots was measured and the leaves number was counted to evaluate the plant's growth performance. The separated shoot and root samples were subjected to drying at 70 °C until a constant weight was achieved and the shoots and roots dry weight was measured.

Drought and inoculation effect on plant physiology. Measurement of stomatal conductance (gs) was conducted in the morning, specifically between 10 a.m. and 12 a.m., on fully developed leaves located at the same height using a leaf porometer (LP1989) (Decagon Device, Inc., Washington, DC, US). To measure chlorophyll fluorescence, an opti-sciences OSI 30P portable fluorometer was used. The *Fv*/*Fm* ratio can be calculated, and it refers to the maximum quantum yield of photosystem II. *Fv* is calculated

by subtracting the minimum fluorescence (*Fo*) from the maximum fluorescence (*Fm*) and then dividing the result by *Fm*. Chlorophyll content was measured according to the protocol described by Arnon [21]. To perform the measurements, 50 mg of plant material was ground and homogenized in 4 cm^3 of 80% acetone. The homogenized mixture was then left in the dark for 2 h to facilitate the extraction of chlorophyll pigments. After the extraction period, the mixture was subjected to centrifugation at 10 000 g for 10 min. Subsequently, the absorbance of the resulting supernatant was measured at two specific wavelengths, 645 nm, and 663 nm, using a double-beam spectrophotometer (VWR, UV--6300PC, China). These wavelengths correspond to the absorption maxima of chlorophyll a and chlorophyll b, respectively. The chlorophyll a (Chl a) and b (Chl b) contents, mg/g FW, were calculated using the formulas:

$$[\text{Chl a}] = \frac{(12.70A_{663} - 2.69A_{645})V}{1000W}$$

$$[\text{Chl b}] = \frac{(22.90A_{645} - 4.68A_{663})V}{1000W}$$

where A_{663} and A_{645} are absorbances at 663 and A645, respectively, V final volume, W weight of fresh leaf sample.

Osmolytes measurement. The Dubois [22] protocol is a widely used method for determining soluble sugars in biological samples. 100 mg of the alfalfa tissues were mixed with 2 cm^3 of 80% ethanol and centrifuged at 5000 rpm for 10 min. The resulting supernatant was used for analysis. 0.25 cm^3 of the extracted solution was mixed with 0.25 cm^3 of 5% phenol and 1.25 cm^3 of concentrated sulfuric acid. The mixture was agitated and allowed to cool for 5–15 min before measuring the absorbance at 485 nm. The soluble sugar content was calculated according to a standard curve generated from a standard solution of glucose (0.2 g/cm^3) using the equation of the line obtained from the standard curve. For protein content, frozen leaf powder samples (0.25 g) were homogenized in a cold mortar with 5 cm^3 of a solution containing 0.1 M sodium phosphate buffer (pH 6.0), 0.1 g of polyvinyl polypyrrolidone (PVPP), and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The mixture was centrifuged at 18 000 g for 10 min at 4 °C to obtain the supernatant, which was used for protein determination and the estimation of activities of different antioxidant enzymes. Total soluble proteins were estimated following the method developed by Bradford [23].

Scavenging enzymes and hydrogen peroxide measurements. Catalase (CAT) activity was determined by measuring the decrease in H_2O_2 concentration at 240 nm over 3 min, following the method by Aebi [24]. The reaction mixture (2 cm^3) consisted of 0.1 M sodium phosphate buffer (pH 6.0), 0.1 mM EDTA, 20 mM H_2O_2, and 0,1 cm^3 of

the enzyme extract. To evaluate superoxide dismutase (SOD) activity, the ability of the enzyme to inhibit the photo-reduction of nitro blue tetrazolium (NBT) was assessed, based on the method developed by Beyer and Fridovich [25]. The reaction mixture contained 2.55 cm^3 of 0.1 mM sodium phosphate buffer (pH 6.0), 75 µL of methionine (55 mM), 60 µL of riboflavin (0.1 mM), 0.3 cm^3 of NBT (0.75 mM), and 50 µL of the enzyme extract. After 30 min of exposure to blue light, the optical density was measured at 560 nm. SOD activity was quantified as enzyme units (EU) per milligram of protein per minute, with one unit defined as the amount of enzyme causing a 50% inhibition of NBT reduction. The H_2O_2 content was determined using spectrophotometry, following the method of Velikova et al. [26]. First, 0.25 g of fresh alfalfa leaves were homogenized in a cold mortar with 5 cm^3 of 10% (w/v) trichloroacetic acid (TCA). The homogenate was then centrifuged at 15 000*g* for 15 min at 4 °C, and the supernatant (0.5 cm^3) was recovered to determine the H_2O_2 content. 0.5 cm^3 of potassium phosphate buffer (10 mM, pH 7) and 1 cm^3 of iodic potassium (1 M) were added to the supernatant and incubated the mixture for 1 h. Then, the absorbance was measured at 390 nm, and the results were plotted against a standard H_2O_2 curve. A blank was used that consisted of 10% TCA in place of the sample extract.

Statistical analysis. The results obtained were analyzed statistically using Statistica software (version 6). A one-way and two-way analysis of variance (ANOVA) was performed to assess the effect of drought stress, inoculation, and their interactions. The Tukey test was used to separate means that were different at $p < 0.05$. Plant growth traits, soil parameters, the examined strains, and their treatment associations were analyzed using principal component analysis (PCA) in OriginPro Software (version 10.00154). By analyzing the results in this way, gain valuable insights into the relationships between our variables and draw meaningful conclusions were given about the factors that influenced the outcomes.

3. RESULTS

3.1. ISOLATION AND CHARACTERIZATION OF PGPR STRAINS

A total of 20 morphologically distinct bacteria that can solubilize the complex phosphate producing a very remarkable clear halo at the time of isolation, were isolated from the rhizosphere soils of the argan tree. Out of these 20 PSB, four efficient strains (LB4, LBP2, LBA8, and LBA19) were selected based on their tolerance to drought stress and PGP abilities. Regarding abiotic stress tolerance, the bacterial strains showed varying levels of tolerance to drought stress caused by PEG 6000 and salt stress caused by NaCl. LBP2 exhibited the highest tolerance to PEG 6000, with a minimal inhibitory concentration MIC > 40%. LBP2 also showed the highest tolerance to salt stress with a MIC

of 2 M NaCl, while LB4, LBA8, and LBA19 showed similar levels of tolerance with a MIC of 1.5 M NaCl (Table 1).

Table 1

Plant growth-promoting traits of selected bacterial strains

Parameter	LBA19	LBA8	LB4	LBP2	RhOL6	RhOL8
Drought	40	30	> 40	> 40	30	30
Salt	1.5	1.5	2	1.5	1.5	1.5
P solubilization, mg/dm^3	1.76±0.5	1.9±0.49	2.43±0.6	1.56±0.62	900.00	1500.00
K solubilization (*DH*/*DC*)	2.06±0.25	1.17±0.8	1.43±0.3	1.77±0.56	–	–
IAA production, µg/cm^3	166.2±24.8	19.4±2.60	30.8±5.3	24.7±4.5	24.44	33.07
Siderophore (*DH*/*DC*)	1.1±0.1	2.5±0.7	0.7±0.08	3.17±0.17	–	–
Nitrogen fixation	+	+	+	+	+	+
HCN production	–	–	–	–	–	–

LBA19 and LBA8 belong to *Streptomyces* sp., LB4 to *Halalkalibacterium* sp., LBP2 to *Bacillus* sp., RhOL6 RhOL8 to *Ensifer meliloti*. *DH*/*DC* is the ratio of the diameters: halo to colony.

All four strains showed the ability to solubilize phosphate, with mean P solubilization ranging from 1.56 to 2.43 mg/dm^3. LB4 exhibited the highest P solubilization while LBA8 showed the highest pH reduction capacity (3.89). Additionally, all strains showed capacity to solubilize complex potassium, with LB4 exhibiting the highest solubilization efficiency (*DH*/*DC*) of 2.06. LB4 showed the highest IAA production level (166.2 µg/cm^3), followed by LBP2 (30.88 µg/cm^3), LBA8 (24.72 µg/cm^3), and LBA19 (19.41 µg/cm^3). LBA8 and LBA19 demonstrated the highest siderophore production capacity with a *DH*/*DC* of 3.17 and 2.5, respectively, which could enhance iron uptake by plants. The overall results suggest that the four bacterial strains have diverse PGP traits and abiotic stress tolerance capabilities, making them potentially useful as bioinoculants for enhancing plant growth and productivity under different environmental conditions.

3.2. GROWTH PROMOTION EFFECT OF SELECTED CONSORTIUM TO MITIGATE DROUGHT STRESS

The inoculation treatment and drought stress had a significant ($p < 0.001$) effect on shoot and root dry weight and leaves number (Fig. 1, Table 2). Water stress had a significant ($p < 0.001$) decrease in shoot and root dry weight, 55% and 56%, respectively. Bacterial inoculation with the selected consortia resulted in a significant increase in the

studied parameters, both under well-watered and stressed conditions. The dry weight of stems and roots of plants inoculated with consortium 3 (T3) was significantly higher under both water stress and normal watering conditions. An increase of 61% was recorded in shoot and root dry weight increase of T3 under stress conditions compared to the un-inoculated control (Fig. 1). Regarding the leaves number, water stress did not have a significant effect under control conditions, while the effect was significant when plants were inoculated with selected consortia. It resulted in a higher leaf number under both conditions and a significant increase in comparison to uninoculated control.

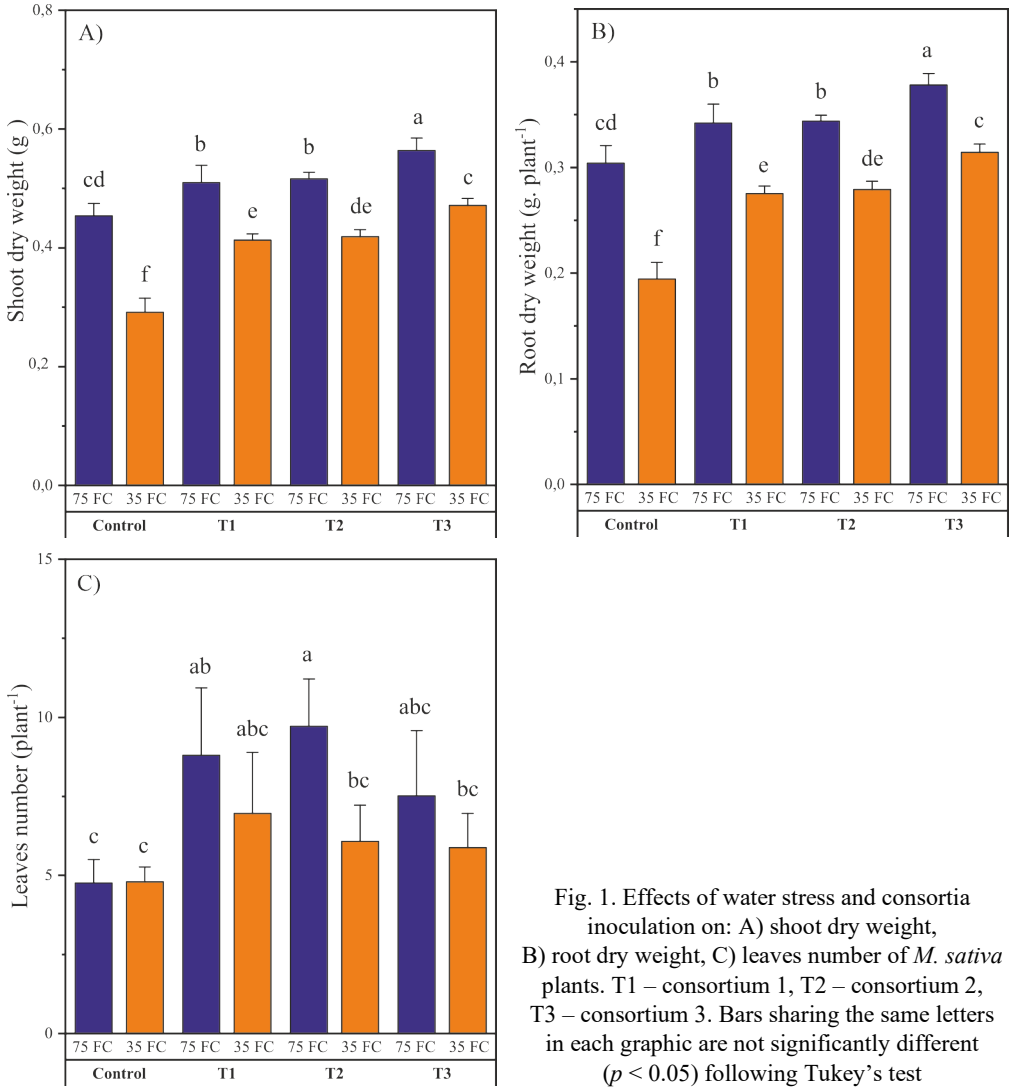

Fig. 1. Effects of water stress and consortia inoculation on: A) shoot dry weight, B) root dry weight, C) leaves number of *M. sativa* plants. T1 – consortium 1, T2 – consortium 2, T3 – consortium 3. Bars sharing the same letters in each graphic are not significantly different ($p < 0.05$) following Tukey's test

3.3. PHYSIOLOGY PROMOTION EFFECT OF SELECTED CONSORTIA TO MITIGATE DROUGHT STRESS

Under WW conditions, bacterial inoculation had a significant ($p < 0.05$) and positive effect on stomatal conductance, chlorophyll fluorescence, and chlorophyll a and b content (Table 2, Fig. 2).

Table 2

Results of two-way ANOVA test for inoculation treatment (Control, T1, T2, and T3) effect, water stress effect (WW and WS), and their interactions

Parameter	Inoculation		Water stress		Inoculation × Water stress	
	F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
Shoot dry weight	103.9	0.000	361.0	0.000	8.0	0.000
Root dry weight	110.4	0.000	395.6	0.000	8.4	0.000
Leaves number	9.5	0.000	13.9	0.000	2.5	0.075
Stomatal conductance	61.9	0.000	69.2	0.000	11.9	0.000
Chlorophyll fluorescence	166.5	0.000	26.2	0.000	12.5	0.000
Chlorophyll a	27.9	0.000	26.6	0.000	3.1	0.054
Chlorophyll b	63.0	0.000	298.1	0.000	19.2	0.000
Sugar content	7.3	0.002	0.9	0.336	5.3	0.000
Protein content	33.8	0.000	1047.5	0.000	0.2	0.883
SOD	59.7	0.000	2118.2	0.000	38.4	0.000
CAT	13.3	0.000	2798.0	0.000	6.6	0.004
H_2O_2	168.1	0.000	1513.7	0.000	151.7	0.000

The obtained results revealed that water stress significantly decreased stomatal conductance compared to normal hydric conditions for both inoculated and control groups (Fig. 2A). However, inoculation with bacterial consortia appeared to mitigate the effect of water stress on stomatal conductance, with consortium 1 and consortium. The percentage increase in stomatal conductance due to inoculation ranged from 74 to 94% under normal hydric conditions and from 23 to 93% under water stress conditions. Regarding chlorophyll fluorescence, the results indicate that bacterial consortia inoculation, water stress, and their interaction had a significant influence (Table 2, Fig. 2B). The obtained results suggested that, in both normal and stressed conditions, consortium 2 exhibited the most significant improvement in chlorophyll fluorescence. Notably, the control group displayed the lowest results in terms of chlorophyll fluorescence under both water stress and normal conditions. Additionally, it is worth mentioning that plants inoculated with consortium 1 and consortium 3 did not display a reduction in chlorophyll fluorescence when subjected to water stress compared to those under normal watering conditions. Moreover, under water stress conditions, the concentrations of both chlorophyll a and b decreased in comparison to the non-stressed group, highlighting the harmful impact of water stress on photosynthetic pigments. Yet, bacterial inoculation partially alleviated this detrimental effect, as evidenced by the higher chlorophyll content

observed in the treated groups when compared to the control group (Figs. 2C and 2D). Inoculating with bacterial consortia led to a notable increase in chlorophyll content, with consortium 2 exhibiting the highest concentration of chlorophyll a, and consortium 3 displaying the highest concentration of chlorophyll b under well-watered conditions.

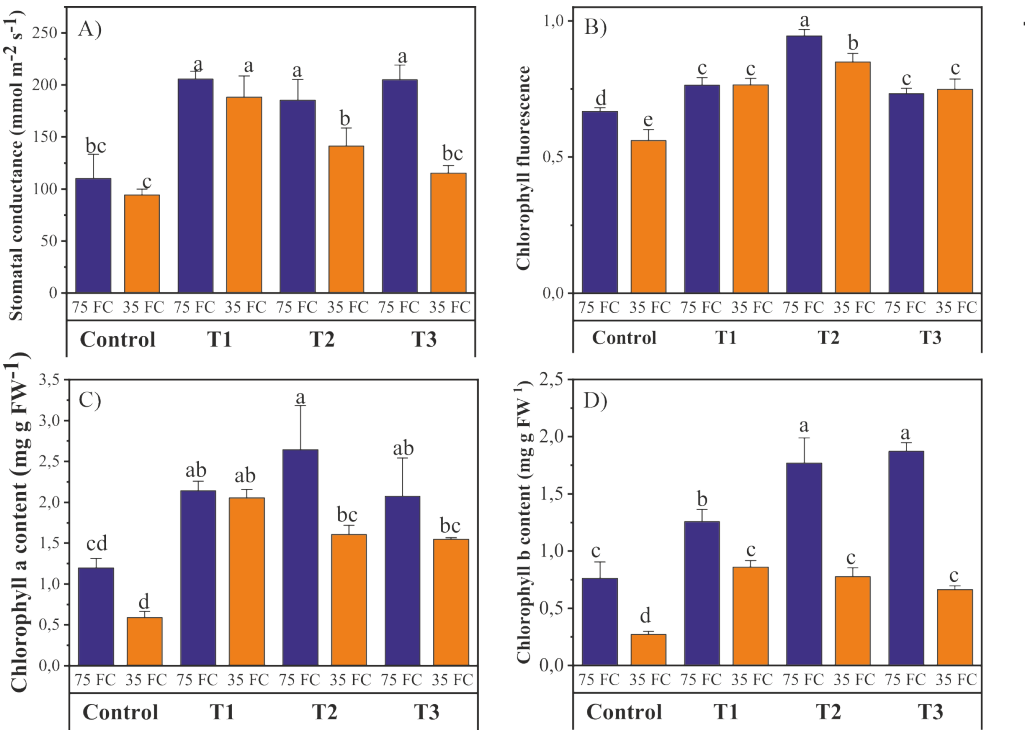

Fig. 2. Effects of water stress and consortia inoculation on: A) stomatal conductance B) chlorophyll fluorescence, C) chlorophyll a, and D) chlorophyll b of *M. sativa* plants contents; T1 – consortium 1, T2 – consortium 2, T3 – consortium 3; bars sharing the same letters in each figure are not significantly different ($p < 0.05$) following Tukey's test

3.4. OSMOLYTES ACCUMULATION EFFECT OF SELECTED CONSORTIUM TO MITIGATE DROUGHT STRESS

Figure 3 shows that the concentration of osmolytes was remarkably higher in stressed plants, whether inoculated or not. However, for both sugars and proteins, under both stress and normal irrigation conditions, plant inoculation was accompanied by an increase in the content of osmoprotective molecules. For sugars, the contents increased from 18.97 to 55.02% for consortia T1 and T3, respectively, under normal irrigation. However, for plants subjected to water stress, this improvement was 45.15, 31.02, and 10.96%, respectively, for T1, T2, and T3 (Fig. 3A). Regarding proteins, the difference in content between inoculated plants was found to be non-significant within the same

water regime (Fig. 3B). It is worth noting that the control groups exhibited lower protein levels compared to the inoculated plants under both well-watered and water-stress conditions (Fig. 3B).

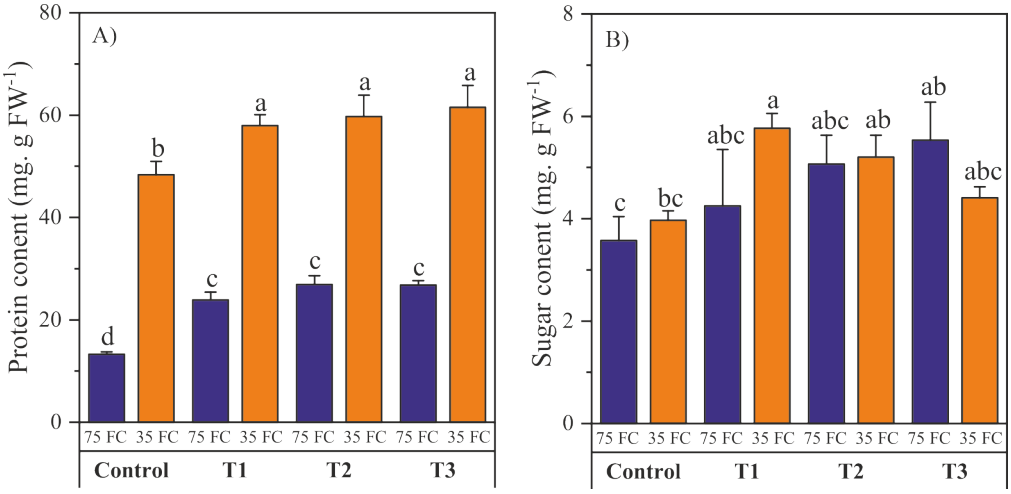

Fig. 3. Effects of water stress and consortia inoculation on: A) protein content and B) sugar content of *M. sativa* plants. T1 – consortium 1, T2 – consortium 2, T3 – consortium 3. Bars sharing the same letters in each graphic are not significantly different ($p < 0.05$) following Tukey's test

3.5. SCAVENGING ENZYMES AND H_2O_2 EFFECT SELECTED CONSORTIUM TO MITIGATE DROUGHT STRESS

The results presented in Fig. 4 demonstrate that the activity of superoxide dismutase (SOD) remained unchanged between the control plants and the inoculated plants under well-watered conditions. However, under water stress conditions, the activity increased for all groups, with the highest value observed for consortium 3, this increase in SOD content is approximately 54.78% compared to the control group. Regarding CAT activity, a considerable increase was observed in the case of alfalfa plants subjected to water stress, reaching up to 286.68% for the control plants while T3 recorded 154.79%. Conversely, the catalase activity did not show a significant difference between the stressed plants. Under normal conditions, the lowest activity was observed in the control plants, while the highest activity in the plants inoculated with consortium 3. However, the results revealed a significant increase in $H2O_2$ concentration when transitioning from well-watered (WW) to water stress (WS) conditions (Table 2, Fig. 4). In WW conditions, H_2O_2 levels remained relatively low without any significant difference observed among all groups. However, under WS conditions, H_2O_2 levels significantly increased for all treatments. Among the inoculated plants, those treated with consortium 3 (T3)

exhibited the lowest H_2O_2 concentration, followed by consortium 1 (T1) and consortium 2 (T2), which showed similar levels.

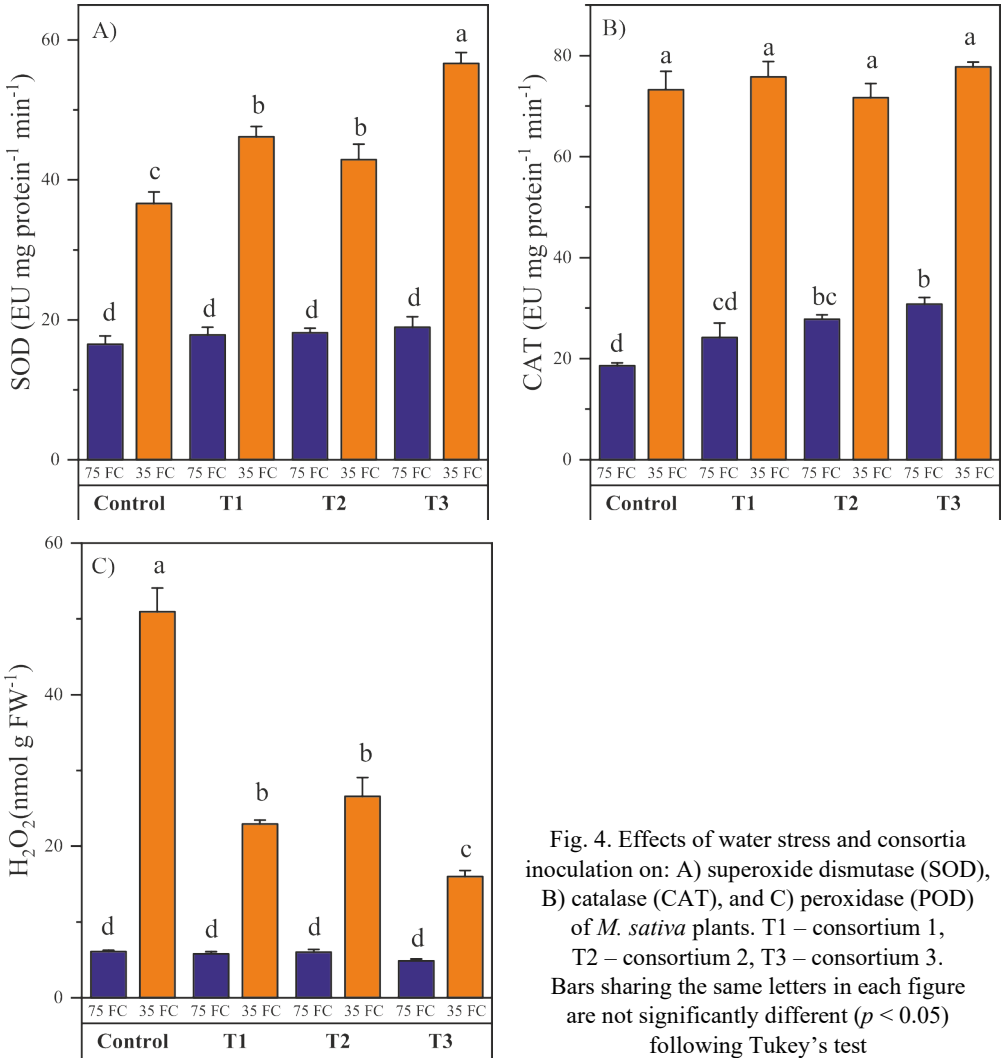

Fig. 4. Effects of water stress and consortia inoculation on: A) superoxide dismutase (SOD), B) catalase (CAT), and C) peroxidase (POD) of *M. sativa* plants. T1 – consortium 1, T2 – consortium 2, T3 – consortium 3. Bars sharing the same letters in each figure are not significantly different ($p < 0.05$) following Tukey's test

3.6. PRINCIPAL COMPONENT ANALYSIS

The investigation conducted by PCA into the interrelationships of the variables under various treatments enabled the identification of two principal components explaining 85.01% of the total variance. The primary component, PC1, illustrated the highest proportion of total variance explained (56.73%), while PC2 displayed the lowest percentage (28.28%) (Fig. 5). The analysis also confirmed the negative impact of drought

on these parameters. Bacterial inoculant treatments were positively linked with growth and photosynthetic traits.

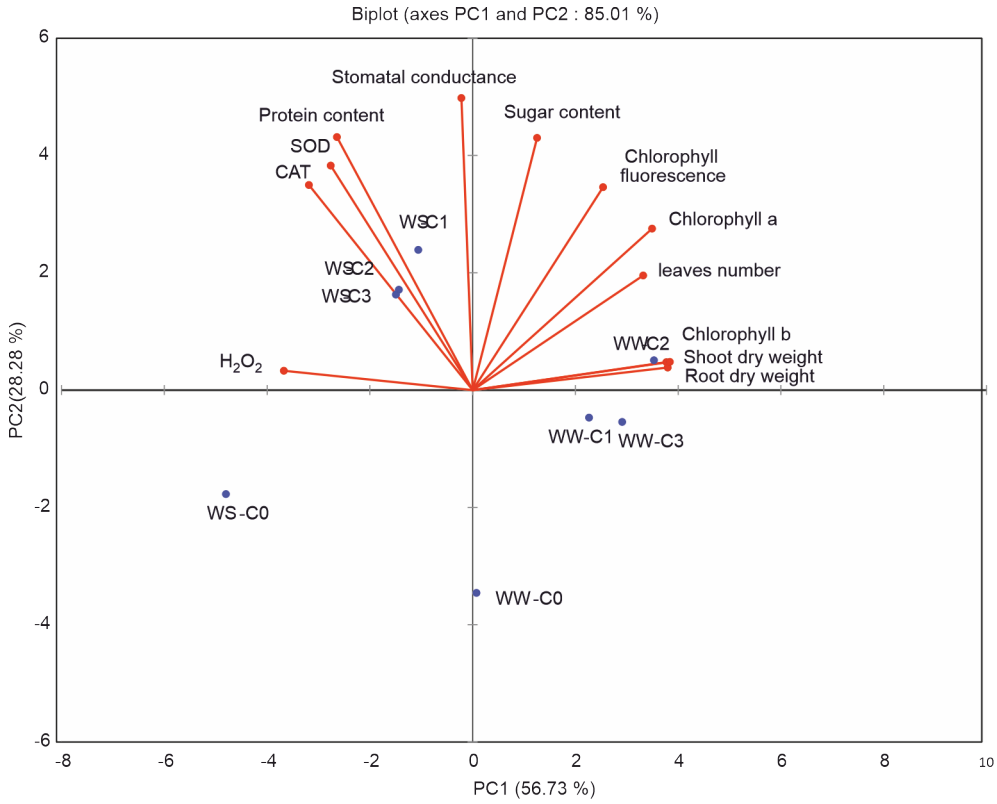

Fig. 5. Results of principal component analysis (PCA) of alfalfa plants submitted to drought stress and inoculated with different rhizobacterial consortia. The treatments are given in red and the parameters are represented in blue. Consortium 1 (C1): RhOL6 (*Ensifer meliloti*) + RhOL8 (*E. meliloti*) + LBA8 (*Streptomyces* sp.) + LBA19 (*Streptomyces* sp.); consortium 2 (C2): RhOL6 (*E. meliloti*) + RhOL8 (*E. meliloti*) + LB4 (*Halalkalibacterium* sp.) + LBP2 (*Bacillus* sp.), consortium 3 (C3): RhOL6 (*E. meliloti*) + RhOL8 (*E. meliloti*) + LBA8 (*Streptomyces* sp.) + LBA19 (*Streptomyces* sp.) + LB4 (*Halalkalibacterium* sp.) + LBP2 (*Bacillus* sp.); WW-C0: Well-Watered uninoculated plants; WS-C0: water stressed uninoculated plants; WW-C1: Well-Watered plants inoculated with the consortium 1; WS-C1: water stressed plants inoculated with the consortium 1; WW-C2: well-watered plants inoculated with consortium 2; WS-C2: water-stressed plants inoculated with the consortium 2; WW-C3: well-watered plants inoculated with the consortium 3; WS-C3: water stressed plants inoculated with the consortium 3; CAT – catalase; SOD – superoxyde dismutase

The treatments of well-watered inoculated plants (WW-C2, WW-C1, and WW-C3) are strongly linked to growth and photosynthesis parameters and are negatively correlated with the antioxidant system. The treatment WS-C0 (water-stressed uninoculated plants) displayed strongly negative values in both dimensions with growth and photo-

synthetic traits and the antioxidant system followed by the treatment WW-C0 (well--watered uninoculated plants). These treatments suggested no association with the variables under study.

The biplot revealed the positive correlation linking drought treatment with H_2O_2 content, the antioxidant enzymes and osmolytes (proteins and sugars), and the bacterial consortia application. The treatments WS-C1, WS-C2, and WS-C3 (water-stressed inoculated plants) were strongly correlated with stomatal conductance, protein content, SOD, CAT, and H_2O_2. Bacterial inoculation improved plant growth under drought conditions in contrast to the Water-Stressed uninoculated plants (WS-C0).

4. DISCUSSION

Drought stress is a significant environmental challenge that negatively impacts plant growth and can lead to substantial crop yield losses. As water availability becomes limited all over the world, specifically in Morocco, plants experience reduced photosynthesis, altered metabolic pathways, and impaired nutrient uptake, ultimately compromising their growth and productivity [4]. Developing approaches to lessen the negative effects of drought stress on agriculture is necessary for ensuring sustainable crop production and food security in countries like Morocco where water scarcity is a pressing issue.

A. spinosa (L.) skeels, an indigenous xerophilic plant species primarily found in Southwestern Morocco, holds significant economic importance due to its production of argan oil, utilized in the cosmetic and food industries. Additionally, it plays a pivotal role in the local agroecosystem, contributing to soil fertility through litter deposition [9, 10]. Despite its ecological and economic significance, there is a notable dearth of research on the microbiota associated with this species, highlighting a crucial gap in the understanding of the role of its bacterial community in ecological interactions and the potential for mitigating desertification. Research on the microbiota associated with the root of *Argania spinosa* indicated their potential as bioinoculants for crops facing water scarcity. For example, in a study conducted by Riva [27] on tomato plants subjected to varying water regimes, certain PGP bacteria isolated from extremophilic plants positively influenced physiological parameters, biomass, fruit productivity, and water use efficiency, suggesting their potential for mitigating water stress in tomato plants. However, gaps exist in the evaluation of the effect of *A. spinosa*-associated microbiota in mitigating the negative effect of abiotic stress on plant growth and development. To our knowledge, this is the first study reporting the isolation of bacteria from *A. spinosa* rhizosphere and evaluating their potential to promote plant growth under drought stress.

The present study concerns the region of Sous-Massa with severe water scarcity and where argan trees are endemic species of the region, will harbor evolved and adapted microorganisms to various environmental abiotic stresses, such as drought. About 20 isolates were isolated from the rhizosphere of the argan tree, and four isolates were chosen

due to their drought and salt stress tolerance and PGP characteristics. While phosphorus is present in abundant quantities in soils, it is mainly found in insoluble forms. Thus, its availability to plants is limited. The obtained results revealed that the selected strains were able to solubilize tri-calcium phosphate. It is well-documented that phosphate-solubilizing bacteria can convert insoluble forms of phosphorus into soluble ones, thereby making them accessible for plant uptake [17]. During this study, the process of phosphorus solubilization in the NBRIP liquid medium was observed to coincide with a decrease in the medium's pH. This suggests that the bacteria employed one of the most prevalent mechanisms, which is organic acid production, to solubilize insoluble phosphorus. This method involves the production of organic acids that detach the metal ions attached to the phosphate, thus liberating it into the solution [28]. However, it is worth noting that no direct correlation was evident between the quantity of solubilized phosphorus and the decrease in medium pH. The selected strains displayed a range of intriguing PGP traits, in addition to P solubilization. They exhibited the ability to solubilize potassium and produce IAA and siderophores. These attributes are widely recognized as indicators of potent growth-promoting capabilities in microorganisms [17]. The production of phytohormones like IAA is of considerable significance as it encourages root development, which subsequently enhances a plant's water and nutrient uptake efficiency. This is a crucial factor in promoting plant growth and resilience under varying environmental conditions [29]. Siderophores, while important for their solubilization activity, also play an essential role in the chelation of Fe^{3+} ions. This activity not only fosters microbial growth but also aids in plant growth by making iron available to plants, thus preventing iron deficiency chlorosis.

Beneficial bacteria have been widely studied for their role in enhancing plant growth and tolerance to drought stress [4]. However, the effectiveness and the interaction of different groups of bacteria with the plant under drought-stress conditions require careful investigation. In this study, *M. sativa* is used as a plant model to gather knowledge on the effect of selected consortium based on regular bacteria, actinobacteria, and rhizobia on the mechanistic of the plant to tolerate drought stress impact and to promote plant growth. The controlled greenhouse experiment demonstrated that inoculation with the prepared consortium significantly improved the resistance of alfalfa plants to water stress. Indeed, regarding the growth parameters, the inoculated plants, whether under water stress conditions or not, exhibited higher biomass production compared to the non-inoculated ones. The water-stressed inoculated plant: WS-C1 (consortia of actinobacteria and rhizobia), WS-C2 (consortia of *Halalkalibacterium* sp., *Bacillus* sp., and rhizobia), and WS-C3 (consortia of all strains) were strongly correlated with stomatal conductance, protein content, SOD, CAT, and H_2O_2. Bacterial inoculation improved plant growth under drought conditions in comparison to the Water-Stressed uninoculated plants (WS-C0). These findings aligned with the results from the study of Chukwuneme et al. [30]. These authors observed the positive effects of PGPR, especially actinobacteria inoculation on stress resistance and growth parameters in maize

plants. The increase in root biomass can be attributed to the ability of plant growth-promoting rhizobacteria (PGPR) to synthesize indole-3-acetic acid (IAA), a hormone responsible for root proliferation. IAA, a crucial auxin, significantly influences the development and elongation of roots, leading to the expansion of fine root networks [31]. This augmentation root system plays a pivotal role in increasing water and nutrient intake from the soil, resulting in improved water assimilation by the plant. Moreover, PGPR may improve plants' ability to directly acquire nutrients with low mobility, such as P, K, N, and Fe found in soil, via phosphate and potassium solubilization, nitrogen fixation, and iron acquisition, all of which have a positive effect in plant growth [6, 29, 30].

Physiological level, stomatal conductance, and chlorophyll fluorescence content were significantly affected by water stress and bacterial inoculation. This decrease in chlorophyll parameters could be caused by a reduction in water supply, the closure of stomata to avoid water loss through transpiration, a decrease in photosynthetic enzyme activity mainly Calvin cycle enzymes, as well as the alteration of the electron transport chain (ETC), which increases ROS within cell organelles, lowering pigment levels and causing disruption of thylakoid structures [32]. However, bacterial inoculation improves physiological parameters to mitigate the negative effects of drought. In line with these results, it has been demonstrated that plants inoculated with PGPR display better relative water content, stomatal conductance, and chlorophyll content, especially in drought-stress situations [4]. This improvement could be related to lowering oxidative stress generated by drought stress, which protects the photosynthetic machinery [33]. Nonetheless, bacterial inoculation can modulate plant hormones, e.g., abscisic acid (ABA) and cytokinin, inducing stomatal opening and enhancing CO_2 uptake and photosynthesis process [34].

The obtained results also demonstrated an increase in protein content under water stress conditions compared to plants under normal water regimes, which may be due to their key role in osmotic regulation under drought stress. In harmony with these results, several studies have shown that plants accumulate proteins as osmotic adjustment substances under drought stress, and their accumulation in plant tissues is directly related to the capacity of plants to mitigate drought [4]. Indeed, the obtained findings showed that protein contents in leaves increased during drought stress in treated plants as compared to uninoculated controls, this suggests that the presence of the bacterial consortia had a beneficial impact on protein production and accumulation, which enhanced the plants' ability to cope with water stress. These results are in line with previous reports demonstrating the utilization of PGPR can play a crucial role in improving sugar and protein contents in numerous plants under water stress [4]. Proteins produced by the PGPR act synergistically with internal plant osmolytes and serve multiple protective purposes, such as maintaining optimal osmotic pressure within cells, preserving cell structural integrity, and countering the oxidative stress induced by water scarcity, consequently maintaining plant growth during drought stress [35].

On a biochemical level, the results clearly showed the impact of water stress and bacterial inoculation on H_2O_2 content in alfalfa leaves. H_2O_2 is a reactive oxygen species

(ROS) that acts as a signaling molecule and is critical in stress responses. In this study, water stress led to a significant increase in H_2O_2 concentration compared to well-watered conditions. This elevation in H_2O_2 levels could be attributed to drought's decisive influence in creating oxidative stress, which disrupts cellular homeostasis and increases the generation of ROS such as H_2O_2 [35]. Under well-watered conditions, no significant difference was observed between the control and inoculated groups. In contrast to these findings, Slimani et al. [4] discovered that PGPR inoculation reduces H_2O_2 levels even in unstressed plants. Otherwise, under water stress conditions, the effect of bacterial inoculation, particularly consortium 3, which consists of a mixture of all selected rhizobacteria, became apparent in H_2O_2 contents. The results indicated that the bacterial inoculation led to a lower H_2O_2 content in the leaves. This decrease in this injury is due to the primordial role of PGPR in neutralizing reactive oxygen species including H_2O_2, by upregulating ROS-scavenging antioxidant compounds and antioxidant enzymatic activities, thereby effectively reducing oxidative stress and enhancing resilience, health, and performance of plants in the face of drought [4].

To counteract the detrimental effects of ROS, plants have evolved a sophisticated antioxidant defense system, which includes various antioxidant enzymes. These enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase, play a crucial role in scavenging ROS and maintaining cellular redox homeostasis [4]. SOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide (H_2O_2), which is further detoxified by CAT and peroxidases. These enzymes act together to minimize the accumulation of ROS and prevent oxidative damage to cellular components, including lipids, proteins, and DNA. Furthermore, antioxidant enzymes also participate in signaling pathways and regulate gene expression under water stress conditions, thereby enhancing plant adaptive responses [36]. Overall, the activity and regulation of antioxidant enzymes are pivotal for plant survival and successful adaptation to water stress conditions. The imposition of alfalfa plants to water stress significantly increased the activity of superoxide dismutase (SOD) and catalase (CAT). However, the inoculated plants exhibited higher SOD activity compared to the control plants, while the catalase activity remained unchanged, which contradicts other studies that have shown an increase in both SOD and CAT enzymes rather than just one following PGPR inoculation [4]. The observed increase in SOD activity suggests that the inoculated plants had a greater ability to scavenge and neutralize superoxide radicals, which are generated under water stress conditions. This enhanced SOD activity may be attributed to the capacity of these rhizobacteria to stimulate the plant defense mechanisms, by an upregulation of SOD production [4].

5. CONCLUSIONS

The selected PGPR strains LBA8 (*Streptomyces* sp.), LBA19 (*Streptomyces* sp.), LB4 (*Halalkalibacterium* sp.), and LBP2 (*Bacillus* sp.) were able to tolerate high levels

of drought (25–40% PEG) and also possessed multiple growth-promoting activities like phosphate and potassium solubilization, and IAA and siderophore production. Inoculation with bacterial consortia prepared from these strains could protect alfalfa from drought by improving photosynthetic machinery, osmolyte accumulation, and antioxidant enzyme activities, and thus plant growth. The finding of our study demonstrated the potential of using phosphate solubilizing bacteria derived from the rhizosphere of the argan tree from the southwestern region of Morocco as a sustainable approach to enhance plants' resilience to water stress. These bacteria could be considered as a promising green fertilizer to enhance plant production under increasing drought conditions.

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