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Isolation and Characterization of Culturable Osmotolerant Microbiota in Hypersaline and Hypergypsic Soils as New Treatment for Osmotic Stress in Plants

Tatiana Gil [1,†], Raquel Teixeira [1,†], André Sousa [1,†], Maria Alice d'Oliveira Palmeiro [1], Alice Cruz Coimbra de Matos [1], Marla Niza Costa [1], María Victoria Ferrer [1], Ana Sofía Rodrígues dos Santos [1], Cristina Sequero López [2], Inês Rebelo Romão [1] and Juan Ignacio Vílchez [1,*]

[1] iPlantMicro Laboratory Oeiras, Instituto de Tecnologia Química e Biológica (ITQB)-NOVA, 2780-157 Lisboa, Portugal; asousa@itqb.unl.pt (A.S.)
[2] GeoBioTec, Department of Earth Sciences, NOVA School of Sciences and Technology, Campus de Caparica, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal
* Correspondence: nacho.vilchez@itqb.unl.pt
† These authors contributed equally to this work.

Abstract: Saline and gypsic soils impede or condition the establishment of farms in many regions worldwide. Stress caused by the accumulation of sodium or calcium ions in the soil drastically limits plant growth and is a limiting factor in the production of many crops. For this reason, saline and gypsic soils were preferentially exploited for mineral extraction. However, nowadays, they can be a source of new biotechnological tools to help in the osmotic stress to which some crops are exposed. In these environments, despite being traditionally characterized by their low biodiversity, we can find well-adapted microbiota that may be able to interact with plants to deal with different environmental stresses. These mechanisms may consist of a very important contribution to the development of new osmotic stress-dealing bioinoculants. The present study sought to elucidate the diversity of the cultivable population of such environments and use them as regulators of soil nutrients and stress-relieving symbionts in plants under osmotic stress. Among the candidate strains selected to cover more scenarios, we found that the strains *Stutzerimonas stutzeri* A38 and *Bacillus pumilus* A49 were able to increase root size under osmotic stress in *Medicago sativa* and *Medicago polymorpha* plants. Moreover, *Peribacillus frigoritolerans* A70 and *Bacillus licheniformis* A46 also enhanced the performance in *M. polymorpha*, showing interesting potential for a future use in wasteland use for production to livestock feeding or other relevant industries.

Keywords: saltpan; osmotic stress; *Medicago*; bioinoculation; soil nutrition

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1. Introduction

Osmotic stress, arising from an imbalance between extracellular and intracellular fluid osmolarity and pressure, is more prevalent in arid or semi-arid climates [1–3]. These conditions, characterized by high temperatures and low rainfall, lead to rapid evaporation, resulting in the deposition of soluble ions such as chlorides (e.g., halites) and sulfates (e.g., gypsum, anhydrites) [4–11]. Among these, the accumulation of calcium chloride in salt soils has the most significant ecological impact [5,12]. Moreover, plants in these regions face constant high osmotic pressure, necessitating specific adaptations in order to keep obtaining nutrients and minimize the stress impact in their development [12,13]. Thus, a soil is deemed saline or salt-stressed when its electrical conductivity exceeds 4 dS/m, with values over 2 dS/m already compromising ecosystem diversity [14], which includes roughly 840 million hectares worldwide affected [15]. Apart from naturally saline areas, certain agricultural and water usage practices (aquifer over-extraction, irrigation with slightly saline water, and seawater intrusion) lead to soil salinization, jeopardizing the food supply

for approximately 1.5 billion people, according to the Global Map of Salt-Affected Soils (GSAS map, v1.0.0; FAO—2021). Most salt-affected regions are concentrated in North, East, and Southern Africa, the Middle East, Central and Western China, Western United States, Central Asia, and Australia [14]. In Portugal and Spain, these regions are predominantly found in the Southeast and Mediterranean areas, Ebro basin, North Meseta, Tagus basin, as well as Alentejo and Algarve [12,16–18]. Both countries have particularly saline zones that serve as accumulation points for exploitable saline resources, such as salt flats or pans. Conversely, gypsic soils, which contain accumulations of gypsum (at least in secondary amounts), cover about 200 million hectares, primarily in very dry environments where evaporation and lack of rainfall lead to gypsum accumulation [9,19,20]. These gypsum-rich soils are widespread in North Africa, the Middle East, Central Asia, and Eastern Australia. In the Iberian Peninsula, the entire Mediterranean coast, especially the Ebro Basin, is rich in this type of soil [21].

Osmotic stress is becoming increasingly prevalent in ecosystems impacted by human activities and areas experiencing advancing desertification. This trend is causing significant annual production losses due to insufficient adaptation time for local plants and crops [2,6,22]. This situation is aggravating if we consider that most crop plants are not selected for their ability to resist osmotic stress, although there are cases of selected genotypes in regions exposed to these conditions. In many cases, domestication led to some species losing their ability to tolerate osmotic stress, exemplified by the contrast between *Solanum pimpinellifolium*, compared to the modern cultivar *Solanum lycopersicum* [23]. On the other hand, gypsic soils pose challenges for cultivating irrigated alfalfa, cotton, wheat, and apricots, according to FAO [11]. In particular, *Medicago* species, relevant forage crops for livestock, are especially interesting given their adaptability and low requirements, supposing an intriguing avenue for land reclamation or revitalizing salinized or depleted soils [24–26]. Nevertheless, different tolerance levels, and the combined impact of climatic and soil stressors often hamper production [22,24,27,28].

The impact of various osmotic stress types is contingent upon the attained intensity, with soil moisture content playing a pivotal role in ion concentration and osmotic equilibrium. Arid conditions heighten osmotic stress in both saline and gypsic soils. Plants under this stress experience detrimental effects from ion accumulation, impeding their growth and development. Systems crucial for nutrient absorption, cell membrane stability, and transport pathways are among the most affected [21,28]. Dysfunction in these systems leads to the buildup of reactive oxygen species and other secondary stressors. Additionally, saline and gypsic soils can induce mechanical issues through crust formation and compaction, negatively impacting root development, water absorption, and seed germination [9,10,21]. Furthermore, salt buildup in specific structures can result in tissue and organ death, though it serves as a prevalent mechanism for mitigating moderate osmotic pressure. With higher intensities, the mechanisms for accumulation and detoxification become more sophisticated, including selective isolation of excess ions or their transformation into less harmful forms. Most plants adapted to salt and gypsum (halotolerant, halophiles, and gypsophiles, respectively) can activate specific antioxidant systems or develop specialized root architectures to counter secondary effects. Another significant set of adaptive mechanisms involves the production of osmoprotectant compounds or compatible solutes [6,29–31].

Despite the substantial challenges posed by saline and gypsic soils, there is an initiation of evaluations regarding their potential for cultivating more adapted species [14]. Moreover, biotechnological strides, including the application of beneficial bacteria, hold promise for revitalizing and enhancing the productivity of these regions. Notably, certain osmoprotectant compounds such as proline and trehalose, which can be synthesized by bacteria, demonstrate the capacity to bolster their production when associated with plants. This serves to safeguard the host and even trigger the host's own production of these compounds [31–35]. Microorganisms play also a crucial role in providing and regulating antioxidants to counteract the reactive oxygen species (ROS) induced in plants during osmotic stress [36–38]. Numerous studies underscored the protective role of bacteria in

the formation of biofilms around plant roots. These structures act as a shield against the toxic effects of accumulated ions and enhance osmotic balance by retaining water at the root surface [37–39]. This protection enables sustained root development. However, certain bacterial strains can modulate root architecture to adopt different strategies against osmotic stress, including increasing the number of secondary roots, restricting their size, or promoting lateral expansion [34,40]. This regulatory process is intricately linked to the production of phytohormones such as auxins, gibberellins, cytokinins, and abscisic acid. Additionally, many strains exhibit the capacity to supply nutrients, including phosphorus, potassium, and metal ions, even under high osmotic pressure in the soil [34,41]. This contributes to improved plant homeostasis and aids in maintaining normal growth rates. Lastly, it is worth noting the production of the enzyme amino cyclopropane carboxylic acid deaminase (ACCd), which modulates ethylene levels [37,41]. This enzyme enables rhizosphere-associated bacteria to utilize ACC as a source of carbon and nitrogen, thereby regulating elevated levels of ethylene and avoiding root architecture deterioration and leaf splitting [36,42].

Hence, harnessing microbiota adapted to high-osmotic-pressure environments is an increasingly promising avenue. Such treatments hold potential for assisting crops in addressing the growing challenge of salinization in agricultural lands affected by factors such as irrigation or marine intrusion [36,37,40,41]. Furthermore, they present a viable approach for reclaiming lands impacted by gypsum accumulation due to evaporation amplified by climate change effects, including increased heat and drought [43,44]. The objective of this study was to pinpoint novel strain inducers of osmotic tolerance in severely stressed environments, including salt flats, salt marshes, and gypsum-rich areas. By screening their capabilities and subjecting various osmotic-sensitive *Medicago* species to tests, we aimed to take the initial steps toward developing new bioinoculants. These bioinoculants could serve to enhance the tolerance and productivity of crop plants in agricultural settings facing analogous osmotic stressors.

2. Materials and Methods

2.1. Sampling

Sampling locations were selected to be recognized as gypsum-rich (≥15% gypsum accumulated under wet conditions), salt-rich areas, with no previous agricultural use, and only used for minerals extraction in some cases. Climatically, excluding Alcochete, which has a moderated Mediterranean, all locations were emplaced as Mediterranean (mid-mountain or coastal). Three samples consisting of 30–50 g were collected in unvegetated areas by using a column collector at a depth of 0–10 cm from 14 locations (Table 1 and Figure 1). They were mixed in order to work with a single sample per location, and when visually present, saline/gypsum crusts were removed in the soil samples. All samples were collected during 2022 and 2023, and stored at 4 °C until processing. Soil samples were characterized based on their pH (as in Mclean, 1983 [15]), their salinity as electroconductivity (EC) by using a YIERYI EC-8801 (Shenzhen, China) device, and their percentage of water content (as in Li and Wang, 2014 [16]; by weight difference after 7 days of incubation at 60 °C).

2.2. Isolation, Identification and Analysis of the Culturable Bacteria Populations in Soil and Roots

Samples of soil (0.25 g) were processed and serially diluted in a sterile 0.45% NaCl solution to isolate culturable bacteria in LB plates (per liter: NaCl, 10 g; yeast extract, 5 g; tryptone, 10 g; and agar, 15 g). The colony-forming units (CFUs) for each sampling point were counted and normalized to the soil dry weight (in mg) of the original sample. Then, morphologically different colonies (following the indications of the American Society for Microbiology [45]) were selected, isolated, and purified. Pure cultures were preserved in 40% glycerol at −80 °C. Thereafter, the isolates were identified by amplification of the hypervariable V5-V8 region (~700 bp) or the full region (~1500 bp) of the bacterial 16S rRNA gene by using the pairs of universal primers 779F (5′-AACMGGATTAGATACCCKG-3′)

and 1392R (5′-GGTTACCTTGTTACGACTT-3′), or 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CTACGGCTACCTTGTTACGA-3′), respectively. Template DNA was extracted from individual colonies by heat shock method [46] and the quality was assessed by spectroscopy with a NanoDrop™ One (Thermo Scientific™, Waltham, MA, USA). PCR was carried out with NZYTaq II 2x Master Mix by Nzytech (Lisbon, Portugal), with this running configuration: initial denaturation phase at 95 °C (2 min); then 40 cycles of denaturation at 95 °C (3 s), annealing at 45 °C (30 s), and elongation at 72 °C (2 min); and a final extension phase 72 °C (7 min). The amplicon bands were assessed by 1% agarose gel electrophoresis by using a Mupid®-exU System (Tokyo, Japan) at 135 V, and by a transilluminator (UVIvue™, UVITEC Company, Cambridge, UK). Here, the sequences were sequenced by GENEWIZ (Leipzig, Germany), and the results were compared to the National Library of Medicine BLAST database to identify the strains (>98% similarity). Strains labeled as 'Unidentified' were not possible to identify (DNA not quality enough or impossible to amplify the fingerprinting genes) after several attempts.

In order to analyze the populations, we prepared a phylogenetic tree with the sequences of the identified strains, aligning with ClustalX2 (v2.1) and visualizing with the iTol drawing tool [47]. Moreover, we also included Shannon and Simpson biodiversity indexes, the index of similarity, the reciprocal index, and the evenness to better understand the diversity and distribution of each sample (Omni Calculator—www.omnicalculator.com/, accessed on 1 June 2023; Virtue—https://virtue.gmbl.se/, accessed on 1 June 2023; and Statology—https://www.statology.org/, accessed on 1 June 2023).

2.3. Strains Growth and Plant Growth-Promoting Traits Performance under Osmotic Stress

2.3.1. Strains Growth under High Osmotic Pressure

Isolated strains were tested under osmotic stress to ensure a proper performance. To do this, we performed a growth assay in a 96-well plate with LB medium for 24 h under continuous shaking (150 rpm) and at 28 °C. Each tested strain was inoculated at optical density (OD_{600nm}) 0.05. The growth was measured using a microplate reader at 600 nm. This, coupled with following determinations, was performed by using 200 mM of NaCl, which was the concentration that we applied to the plant test as osmotic-stressing condition, according to different sensibility levels reported previously for different *Medicago* species [48,49]. This will ensure the performance of the different skills under saline stress. In this sense, to determinate the skill performance in our strains, we followed the dynamic semi-quantitative assessment in a solid medium screening, followed by a quantification in liquid medium for the best candidates, maintaining the conditions explained above.

2.3.2. Nitrogen Fixing Activity

The nitrogen fixation activity was assessed following the indications of Sulistiyani and Meliah, based on Bromothymol Blue (BTB) (100 mg/L) as the indicator [50]. In this assay, strains were grown in Jensen's agar medium (per liter: 20 g of sucrose, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, 0.5 g of NaCl, 0.1 g of Fe_2S0_4, 0.005 g of $Na_2MoO_4{\cdot}2H_2O$, 2 g of $CaCO_3$, and 15 g of agar) for 7 days. In positive isolates, the dark blue-colored halos were measured, and the relative nitrogen fixation ability was calculated with respect to the colony size to normalize the results. For quantification, strains were grown in liquid Jensen's amended with BTB (150 rpm, 28 °C) for 3 days, and the absorbance was measured at 640 nm [51,52]. The nitrogen-equivalent standard curve was prepared with NH_3.

2.3.3. Phosphate and Potassium Solubilization

Beginning with the phosphate-solubilizing performance in our strains, we followed Zheng and collaborators, with slight modifications [53]. Thus, a 10 µL drop of each culture was placed on NBRIP agar medium (per liter: 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 5 g of $MgCl_2{\cdot}6H_2O$, 0.25 g of $MgSO_4{\cdot}7H_2O$, 0.2 g of KCl, 0.1 g of $(NH_4)2SO_4$ and 15 g of agar), and the plate was incubated for 5–7 days at 28 °C. Halos produced were measured. In the case of the evaluation of potassium solubilization, we employed Aleksandrov medium

(per liter: 5.0 g of glucose, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCO_3$, 0.006 g of $FeCl_3$, 1.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, 3.0 g of potassium aluminum silicate (mica), and 15 g agar; pH 7.2), amended with BTB (100 mg/mL), according to Rajawat and collaborators [54]. For quantification, we incubated the strains in the respective media in liquid format for three days. In the case of the phosphate solubilization test, the plates were centrifuged (4000 rpm, 10 min) and the supernatant was mixed with 3,5:1 (*v*:*v*) vanadate-molybdate reagent. After incubation in the dark for 10 min, the absorbance was measured at 420 nm. A phosphate standard curve was constructed using anhydrous KH_2PO_4. For the potassium solubilization test, after the initial grow incubation, samples were measured at 430 nm. Standard curve was performed based on Rajawat formula.

2.3.4. Sulfur-Oxidizing Activity

The sulfur-oxidizing skill is going to be especially relevant for the strains isolated from gypsic soils. We prepared this test according to Hidayat, Saud, and Samsudin, with some modifications [55]. A drop of each cultured strain was placed on thiosulfate mineral medium (TSM) (per liter: 1.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, 0.4 g of NH_4Cl, 0.8 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 10 g of $Na_2S_2O_3 \cdot 5H_2O$, and 15 g of agar; pH 7.5), with 0.01 g of bromocresol purple, and incubated for 14 days at 28 °C. After measuring the discoloration halos, the quantification was prepared in TSM liquid medium and grown for 14 days at 28 °C and 160 rpm. The centrifuged supernatant (4000 rpm, 10 min) was mixed 1:1 (*v*:*v*) with barium chloride ($BaCl_2$) solution (10% *w*:*v*) and the absorbance of the resulting mix was measured at 480 nm. The standard curve was performed by dissolving potassium sulfate (K_2SO_4) in a $BaCl_2$ solution.

2.3.5. Siderophores Production

The siderophore production was assessed following the indications of Arora and Verma, with minor modifications. In brief, a drop of each cultured strain was placed on blue agar chrome azurol sulfonate (CAS) plates [56,57]. A yellowish halo around the strain was considered as positive and measured. Then, a regular LB culture of each strain was centrifuged (4000 rpm, 10 min), and 100 µL of the supernatant was mixed with 100 µL of CAS reagent in a 96-well plate. After 20 min of incubation at room temperature, absorbance was recorded at 630 nm. Siderophore production was calculated as the percent siderophore unit (psu): psu = (absorbance control – absorbance sample) × 100 absorbance control.

2.3.6. Biofilm Production

Biofilm production was evaluated as in Coffey and Anderson [58], with slight modifications. Briefly, strains were inoculated in LB medium in a 96-well plate at 28 °C and 150 rpm. Then, planktonic structures were washed and only biofilm structures were stained with 200 µL of 0.2% crystal violet solution. After incubating for 20 min at room temperature, the excess crystal violet was washed and structures were solubilized with 30% glacial acetic acid solution for 20 min. Finally, the solution was measured at 550 nm. In this test, together with auxins and ACC deaminase production, we used *Pseudomonas putida* KT2440 values as reference (positive control).

2.3.7. Auxins Production

For auxin production, we followed the method described by Ambrosini and Passaglia [59]. Thus, 200 µL of LB medium supplemented with tryptophane (0.5 g/L) were inoculated with each strain and incubated for 48 h at 28 °C and 150 rpm. After centrifuge (4000 rpm, 30 min), 100 µL of supernatant were mixed with 100 µL of Salkowski reagent (0.5 M of $FeCl_3$ and 35% $HclO_4$). Finally, after a 30-minute incubation at room temperature in the dark, the absorbance was measured at 530 nm. The values were determined as indole-3-acetic acid (IAA) equivalents with respect to a calibration curve.

Table 1. Sampling locations. Samples were collected in different locations of the Iberian Peninsula, defined by numbers on the map (Figure 1) and their own name (with sample acronym in parenthesis). The locations were situated in Spain (SP) and Portugal (PT). Samples were collected in saline and gypsic soils in a 0–15 cm depth. GPS coordinates (World Geodetic System 1984, WGS84) and altitude (meters above mean sea level, mamsl) are approximated. Geologic context, soil type, and soil variables (pH, electro-conductivity) are determined as factors conditioning the microbiota.

No.	Type	Name	Location	GPS	Altitude (Mamsl)	Main Basal Lithology [a]	Type of Soil [b]	pH [c]	EC [c,d]
1	Gypsum	Arroyo Salado (AR)	La Malahá, Granada (SP)	37°06′25.3″ N 3°43′14.7″ W	726	Dolomites and dolomitic marbles	Cambic calcisol	8.02	0.31
2	Gypsum	Rambla de la Mojonera (RM)	Sorbas, Almería (SP)	37°05′59.5″ N 2°08′08.7″ W	395	Sands and gravels	Cambic calcisol	9.79	0.06
3	Gypsum	Hoya de Baza (HB)	Baza, Granada (SP)	37°30′50.4″ N 2°45′34.5″ W	792	Clays with pebbles	Calcaric fluvisol	8.52	0.4
4	Gypsum	Quinto (GQ)	Quinto, Zaragoza (SP)	41°26′51.8″ N 0°32′15.5″ W	195	Gypsum, marlstones limestones	Haplic calcisol	8.46	0.52
5	Gypsum	Zuera (GZ)	Zuera, Zaragoza (SP)	41°52′56.7″ N 0°47′21.6″ W	335	Gypsum and clays	Haplic gypsisol	8.56	0.18
6	Salt	Salina de San José (SJ)	Torredonjimeno, Jaén (SP)	37°45′26.8″ N 4°00′12.1″ W	451	Marlstones and marly siltstones	Cambic calcisol	8.66	2.08
7	Salt	Salinas de las Escuelas (SE)	Baeza, Jaén (SP)	37°52′15.9″ N 3°31′18.1″ W	479	Dolomites	Cambic calcisol	9.58	2.16
8	Salt	Salinas de Las Cañadas (SC)	Montejícar, Jaén (SP)	37°36′31.9″ N 3°30′19.9″ W	1028	Conglomerates, sandstones, clays	Cambic calcisol	8.64	2.18
9	Salt	Laguna Roja (LR)	Torrevieja, Alicante (SP)	37°59′46.8″ N 0°42′11.8″ W	−2	Siltstones	Calcaric leptosol	9.24	2.68
10	Salt	Saladas de Sástago (SB)	Bujaraloz, Zaragoza (SP)	41°25′15.6″ N 0°11′39.6″ W	322	Clays and silstones	Calcaric fluvisol	8.22	2.42
11	Salt	Saladar del Baíco (EB)	El Baíco, Granada (SP)	37°32′28.5″ N 2°43′57.1″ W	711	Marls, conglomerates, limestones, sandstones	Cambic calcisol	8.97	5.37
12	Salt	Salinas do Samouco (SS)	Alcochete, Setúbal (PT)	38°44′11.6″ N 9°00′01.7″ W	0	Sandstones and conglomerates	Eutric regosol	6.88	2.23
13	Salt	Barranco de las Salinas (BS)	Gádor, Almería (SP)	37°01′01.9″ N 2°26′51.7″ W	263	Marls, sandstones and siltstones	Cambic calcisol	9.38	1.94
14	Salt	Rambla de Librilla (RL)	Librilla, Murcia (SP)	37°54′23.7″ N 1°22′15.8″ W	205	Conglomerates and sandstones	Cambic calcisol	9.21	3.78

[a] Source for "Litology of basement" in Spain (SP): Instituto Geológico y Minero de España (IGME), MAGNA escala 1:50.000 (year of the geological cartography between 1973 and 1992); in Portugal (PT): Carta dos solos do Portugal (1971) escala 1:1.000.000, Secretaria de Estado da Agricultura, Serviço de Reconhecimento e de Ordenamento Agrário; [b] Source for "Type of soil": Atlas Nacional de España, Instituto Geográfico Nacional (based on European Soil Data Centre (ESDAC). Eurpean Commission, 2001). 'Undif.' abreviation stands for undifferentiated geological context; [c], values of samples collected in the 0–10 cm-deep fraction; [d], 'EC' stands for electric conductivity measurement.

2.3.8. Production of 1-Aminocyclopropane-1-Carboxylate Deaminase (ACCd)

The production of 1-aminocyclopropane-1-carboxylate deaminase (ACCd) was evaluated by following the previously adapted methodology [60]. Briefly, using a 96-well plate, the strains grew in M9 medium (Merck) amended with 3 mM of ACC, and after 24 h at 28 °C and 150 rpm, the culture was measured at 600 nm in order to evaluate the culture

growth and compare among the collection of strains. This measure gave us an indirect confirmation of the ACCd production by each strain.

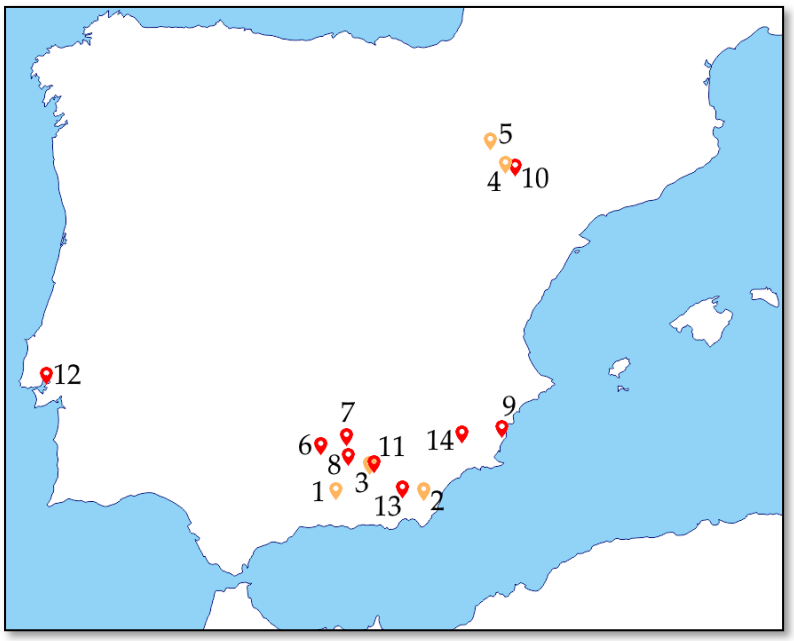

Figure 1. Sampling locations. The map shows the locations selected for sampling (the numbers refer to the locations listed in Table 1) along the Iberian Peninsula. Location pins in yellow indicate samples collected in gypsic soils; red pins indicate samples collected in saline soils.

2.3.9. Antioxidants Production

We began by detecting catalase activity following the indications of Reiner [61], which consisted of dropping 3% hydrogen peroxide over a fresh colony placed on a microscope. Bubble formation was considered as positive in catalase, and the quantification was performed by strictly following Hadwan protocol [62]. Then, for general antioxidants production, we followed the thiocyanate method, as described by Takao and collaborators [63]. In brief, 200 µL of centrifuged (8000 rpm, 5 min) overnight-cultured strain was mixed with 200 µL linoleic acid solution (25 mg/mL in pure ethanol), 400 µL of phosphate buffer (per liter: 20.214 g of $Na_2HPO_4{\cdot}7H_2O$, 3.394 g of $NaH_2PO_4{\cdot}H_2O$; pH 7.0), and 200 µL of double distilled water. The samples were incubated at 40 °C for 10 min in the dark. Subsequently, 100 µL were mixed with 3 mL of 75% ethanol, 100 µL of NH_4SCN solution (0.3 g/mL in double distilled water), and 100 µL of ferrous chloride reagent (2.45 mg/mL of $FeCl_2$ in 3.5% hydrochloric acid). Finally, after 3 min of incubation at the root temperature, absorbance was measured at 500 nm.

2.4. Plant Material: Medicago Species

To carry out plant tests of the candidate strains, we decided to use different species of *Medicago*. Within this genus, we can find species and varieties with diverse responses to osmotic stress. We selected species with different tolerance levels of *M. sativa* (sensitive) and *M. polymorpha* (mid-tolerant) as model plants. Seeds were commercially available (Cantueso Natural Seeds, Córdoba, Spain) or were collected from nature in different regions of Portugal and Spain during spring/summer 2022 [60].

2.5. Germination and In Vitro Colonization Tests under High Osmotic Preassure

The germination test was carried out following the method described in Niza and collaborators for *M. sativa* [60]. Briefly, 100 seeds per plant species were surface-sterilized (70% ethanol, 5 min; 3× sterile double distilled water (ddH_2O) washing) and incubated in water 48 h at 4 °C to enhance the germination rate. Each set of seeds was then soaked in a

bacterial culture in 0.45% NaCl solution (10^8 CFUs/mL) or in bacteria-free saline solution in the case of the mock set of seeds. Next, the seeds were placed in Magenta boxes over wet towel paper with or without 200 mM NaCl as a stressing factor. After 7 days of incubation in the dark, the germination rate (%) was calculated based on bacterial treatment and conditions. This experiment was repeated three times.

On the other hand, the in vitro colonization tests were prepared following the indications of Vilchez and collaborators, with slight modifications to cope with the type of plant and applied stress [64]. Hence, five seedlings per replica were surface-sterilized as indicated above and placed on Phyto agar plates (Duchefa Biochemie). After 7 days of incubation, the roots of the germinated seedlings were placed in 1.5 mL tubes with a bacterial solution (10^8 CFUs/mL) and incubated overnight with agitation (150 rpm) at 28 °C. An additional set of samples was prepared by increasing the osmolarity (200 mM of NaCl) as stressing treatment. Thereafter, the roots were surface sterilized in 70% ethanol and washed with sterile ddH_2O before grinding and serial dilution. The dilutions were placed on LB plates and incubated for 24 h at 28 °C. Finally, CFUs were quantified and normalized to the root dry weight of the original sample. The differential rate of colonization between the treatments with and without osmotic stress was recorded for each bacterial treatment. Each treatment was repeated three times.

2.6. Evaluation Plant Osmotic-Tolerance Enhancement

For test in pots, seeds were initially surface sterilized as previously indicated. Once in magenta boxes, they were placed in darkness at 22 °C for 2–3 days. Then, the seedlings were transferred to 0.5 L pots full of a turf:natural soil:vermiculite (2:1:1, *v*:*v*) mix. Pots were maintained in the greenhouse one day for acclimation. Thereafter, the seedlings were grown for 7 days and then inoculated with 40 mL/pot of each selected candidate strain (10^8 CFUs/mL ($OD_{600nm} \approx 1.0$) in sterile 0.45% NaCl). For the control set (mock), seedlings were inoculated with 40 mL of bacteria-free, sterile 0.45% NaCl. After two days, a set of plants (25 plants per treatment) was irrigated with 200 mM NaCl saline solution as an osmotic-stressing factor. Fifteen days after treatment (DAT), the test was ended and the phenotype recorded. Here, we included the root length, shoot height, and total dry weight (DW). EZ-Root-VIS (v2.5.4.0) and ImajeJ (v1.54e) softwares were used to standardize and analyze the measures [65,66].

2.7. Statistics

The statistical analyses were performed in Prism (v9.0.0, GraphPad Software, Boston, MA, USA). Here, we used Student's *t*-test or two-way ANOVA (with Tukey's and Šidák tests). The significance level was set at $p < 0.05$ for biodiversity analyses. Moreover, we used online tools from Virtue (virtue.gmbl.se/english-content/biodiversity-calculator, Gothenburg Marine Biological Laboratory, Gothenburg, Sweden) for biodiversity indexes. For the PCA analyses, we used the Principal Component Analysis Calculator from Statistics Kingdom (statskingdom.com/pca-calculator.html, Melbourne, Australia). All the online tools were used in their versions for May 2023.

3. Results

3.1. Population Analysis

A total of 104 strains were isolated from the 14 locations of gypsum-rich soils (1–5), and salt-rich soils (saltpans and salt flats; 6–14) (Table 2 and Figure 2). We were able to identify 74 strains, and all sequences were submitted and are accessible in GenBank through the accession number OQ971766-OQ971803. However, about 35% of the population in gypsum-rich soils on average, and less than 2% in the salt-rich ones, remained unidentified. The raw data and analysis of populations and screening tests presented here are openly available in FigShare [67]. The populations of the different samples, despite being quite variable as mentioned above, were similar in terms of average colony-forming units (CFUs) per mg of soil (dry weight), with populations around 2.06×10^2 and 2.12×10^2 in gypsum-

and salt-rich soil samples, respectively. Considering the strains by origin, we can observe that 15 of them were uniquely isolated from gypsum-rich soils (14.4%), 69 uniquely from salt-rich soils (66.4%), and 20 strains were found in both environments (19.2%).

Table 2. Strains collection. This table shows the strains isolated and identified in the 14 locations covered by this study. Some species were isolated in different locations, so each location number where we found them is included here.

Species Isolated	Location
Rhizobium zeae	2
Pseudarthrobacter oxydans	1, 6
Arthrobacter globiformis	11
Arthrobacter agilis	6
Pseudomonas sp.	1, 2, 6
Pseudomonas fragi	6
Pseudoclavibacter helvolus	6, 7, 8
Stutzerimonas stutzeri	6, 7, 11
Pantoea agglomerans	10
Leclercia adecarboxylata	1, 6, 8
Bhargavaea beijingensis/cecembensis	14
Streptomyces spiroverticillatus	3, 9
Niallia circulans	11
Niallia nealsonii	11
Bacillus cereus	13
Bacillus pumilus	9, 11, 13
Bacillus subtilis	5, 10
Bacillus thuringiensis/toyonensis	2, 14
Bacillus thuringiensis	10
Bacillus atrophaeus	2, 9, 10, 11, 13, 14
Bacillus mojavensis	3, 11
Bacillus safensis	9
Bacillus sonorensis	13
Bacillus licheniformis	2, 9, 11, 13, 14
Peribacillus frigoritolerans/simplex	1, 2, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14
Peribacillus muralis	6
Paenibacillus amylolyticus	2, 14
Paenibacillus taichungensis	10,12
Paenibacillus urinalis	11
Paenibacillus polymyxa	2, 13, 14
Priestia endophytica	11
Priestia megaterium	12
Priestia flexa	11
Metabacillus idriensis	2, 14

The predominant family within the collection was *Bacillaceae* (almost 3/4), being consistently the more prevalent in both sampling environments (Figures 2 and 3). Most of the

strains belong to the group of species *Peribacillus frigoritolerans*, *P. simplex* and *P. muralis*. These strains were more frequent and prevalent in salt-rich soils. The next species by presence was *Bacillus atrophaeus*, with six strains, being more relevant in salt-rich soils. Moreover, two genera gathered four strains each, *Priestia* and *Paenibacillus*. Finally, another 12 species were found belonging this family: *B. licheniformis* (4), *Bacillus subtilis* (2), *B. thuringensis/toyonensis* (3), *B. pumilis* (2), *B. mojavensis* (2), *B. cereus* (1), *B. safensis* (1), *Bacillus sonorensis* (1), *Niallia nealsonii* (1), *N. circulans* (1), *Metilobacillus idriensis* (1), and *Bhargavaea beijingensis/cecembensis* (1).

The next family in abundance was *Pseudomonadaceae* (about 12%), founding five strains identified as *Stutzerimonas stutzeri* (all of them in salt-rich soils), and four as *Pseudomonas* sp. (more prevalent in gypsum-rich soils). The family *Micrococcaceae* was represented with three species (*Pseudoarthorbacter oxydans*, *Arthrobacter agilis,* and *A. globiformis*), mostly found in salt-rich conditions. On the other hand, two strains, *Leclercia adecarboxylata* and *Pantoea agglomerans*, were the only representation of the order Enterobacterales. Finally, some other strains were identified, but were barely represented among the isolates (*Pseudoclavibacter helvolus*, *Streptomyces spiroverticillatus*, and *Rhizobium zeae*). The analyses of the biodiversity indexes did not show any significant differences between the values of the samples by origin.

3.2. Characterization and Selection of Candidate Strains

All the isolated strains were tested in order to characterize them as candidates for plant tests (results are openly available in FigShare [67]; (Supplementary Figure S1)).

Thus, we included tests to assess plant growth promotion and stress tolerance enhancement traits, which will be highly valuable in osmotic stress dealing. Hence, we analyzed the auxins production, where we found 25 strains able to produce above 25 μg/mL, highlighting the strains *Stutzerimonas stutzeri* 21 (51.32 μg/mL) and *Peribacillus frigoritolerans* A28 (58.415 μg/mL), isolated from salt-rich soils, but especially *Pseudomonas* sp. BABY−A48 (95.44 μg/mL), isolated from gypsum-rich soils. Here, more than 70% of the strains were only happening in salt environments. On the other hand, the production of ACC deaminase activity was indirectly assessed by growth with ACC as the sole N and C source. Thus, almost 70% of the strains showed a representative growth (71), with 14 growing above 0.8, as *Bacillus licheniformis* A46, *Pseudomonas* sp. BABY−48, and *Bacillus cereus* A74. These strains were again more present in salt-rich soils (65%) than in gypsum-rich ones (less than 14%). Another relevant ability considered here was the biofilm production. Here, almost 50% of the strains were able to produce above 0.54 units (P. putida KT2440 reference value). The strains *Stutzerimonas stutzeri* A38, *Arthrobacter globiformis* A32, *Priestia flexa* A25, *Stutzerimonas stutzeri* A30, *Stutzerimonas stutzeri* A31, and *Bacillus thuringiensis/toyonensis* A64 were the most remarkable in biofilm production. From the producers, 67% were only isolated from salt-rich soils and 12% from gypsum-rich soils. Finally, we evaluated the production of antioxidants, where 10 strains were able to produce above 6 mM of antioxidants equivalents (as *Peribacillus simplex* 13 or *Bacillus pumilus* A49). From the strains that were able to produce antioxidants, only 55% was present in salt-rich soils, and 29% was unique from gypsum-rich soils.

Following with nutrient related traits, some of them were more prevalent in strains isolated in salt-rich soils (as nitrogen fixation, P and K solubilization, sulfur oxidizing, and auxins and biofilm production). However, there were still many strains identified in both environments, so we decided to evaluate the prevalence after evaluating the clustering degree of the data in a principal component analysis (PCA) (Figure 4). All the analyzed groups showed high overlapping clustering, indicating no relevant differences among them. The dispersion of the results only allowed us to discern that most of the data aggregate around the traits analyzed. Among them, sulfur oxidizing, nitrogen fixation, and the solubilization of phosphorus and potassium were shown to be more correlated than the rest of the traits, indicating the common presence of these capabilities in most of the strains evaluated. On the other hand, ACCd, biofilm, and auxins formed a second

group of abilities, inverse in respect to the previous one, but not that cohesive, indicating a less-regular appearance of these three skills at the same time and along the strains.

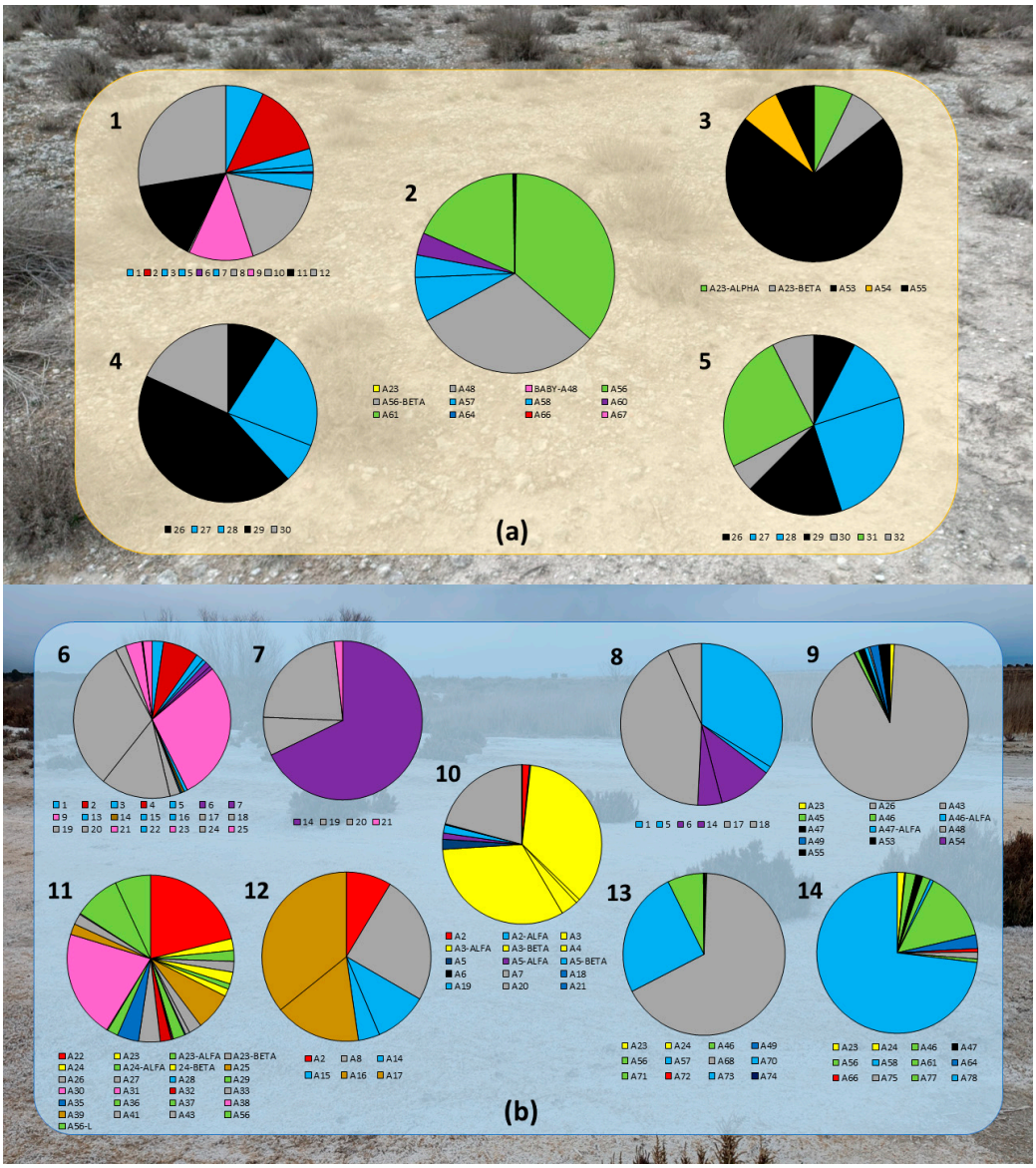

Figure 2. Population distribution. The panels with pie charts represent the population distribution (relative abundance) in each sample point (numbers correspond to the one mentioned in Table 1) in gypsum-rich (**a**) and salt-rich (**b**) soils. The colors of the pie charts were selected in order to compile closely related species or taxa. Thus, the sector of the pie charts in light blue stands for the group of *Peribacillus frigoritolerans*, *P. simplex* and *P. muralis*; in dark blue, for the group of *Bacillus subtilis*, *B. thuringensis*, *B. cereus*, *B. pumilis*, and *B. toyonensis*; in yellow, for the strains identified as *Bacillus atrophaeus*; in red, for the strains from genus *Paenibacillus*; in brown, for the strains from the genus *Priestia*; in green, for the rest of the strains from the family *Bacillaceae* (*B. mojavensis*, *Niallia nealsonii*, *N. circulans*, *B. licheniformis*, *Metilobacillus idriensis*, *B. safensis*, *Bhargavaea beijingensis/cecembensis*); in pink, for the strains from the family *Pseudomonadaceae* (*Pseudomonas* and *Stutzerimonas*); in deep red, for the strains from family *Micrococcaceae* (*Arthrobacter* and *Pseudarthobacter*); and in purple, for the rest of the less represented strains (*Leclercia*, *Pseudoclavibacter*, *Pantoea*, *Streptomyces*, and *Rhizobium*). Moreover, the unidentified strains are represented in black-colored sectors when they showed fungi or yeast morphology, and in grey for other strains.

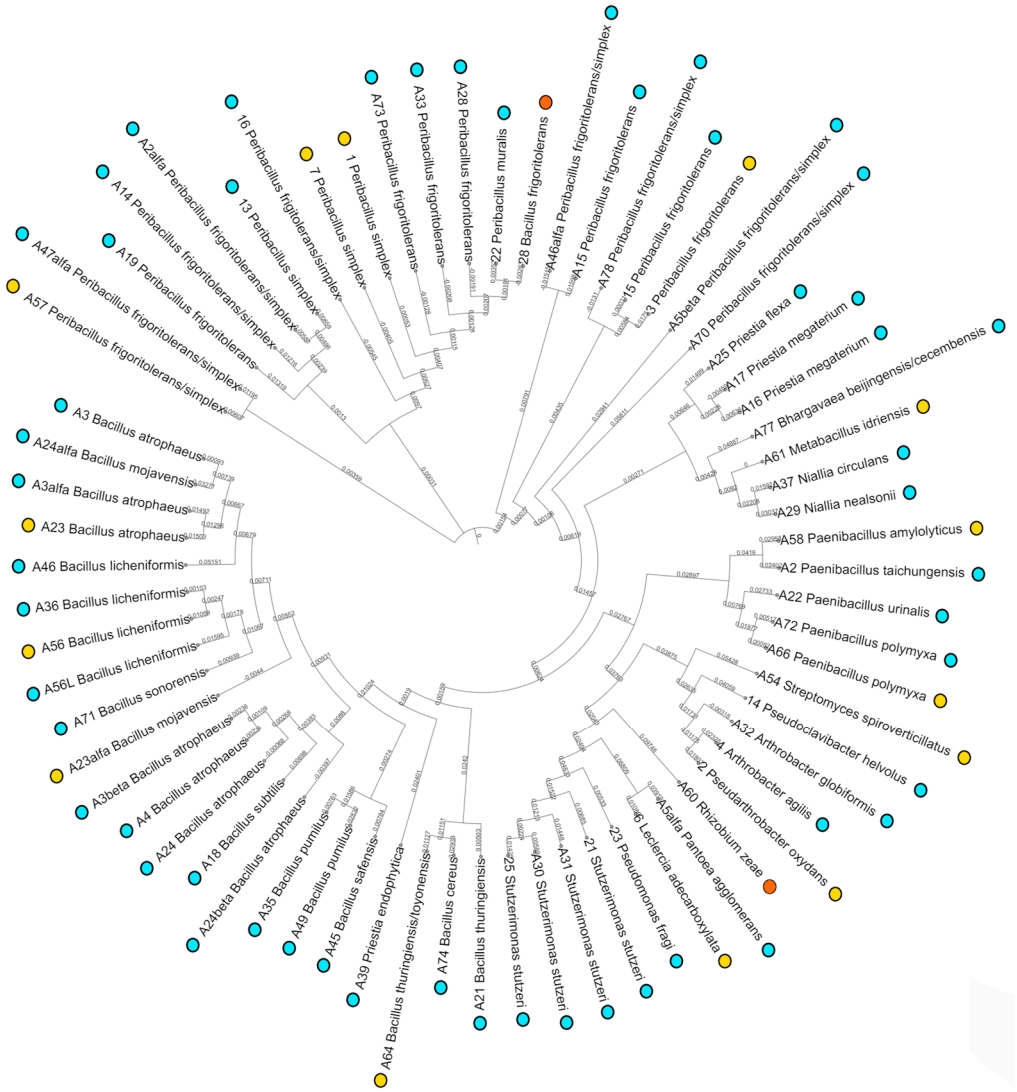

Figure 3. Phylogenetic tree. The circular phylogenetic tree shows the proximity of the strains isolated in this study, indicated by branch length. The strains labeled in blue were exclusively isolated from hypersaline soils; the ones labeled in orange, exclusively from hypergypsic soils; meanwhile, the ones labeled in yellow, from both sources. Distances between strains are numerically indicated in the tree branches.

3.3. Strains Selected for Osmotic Stress Tolerance Treatment

Due to the high diversity of strains isolated, as well as their different performances under each test, we decided to evaluate a total of five strains. In this sense, the selected candidates were the identified strains with better performance for each of the tests, but in some cases, they were shown as top performers in more than one test. Thus, the strain *Stutzerimonas stutzeri* A38 was selected as a nitrogen fixator and sulfur oxidizer; *Peribacillus frigoritolerans/simplex* A70 as a P and K solubilizer and siderophores producer; *Niallia circulans* A37 as a biofilm and auxin producer; *Bacillus licheniformis* A46 as an ACC deaminase producer; and *Bacillus pumilus* A49 as an antioxidant producer. Each strain was then prepared as a different strategy or approach to evaluate as biotreatment in order to enhance plant tolerance to osmotic stress. Moreover, we performed a precise quantification of the performance of these strains in a 96-well high-throughput system, which allowed us to include osmotic stress as a treatment. The stress level was fixed to 200 mM of NaCl to cope with the stressing conditions that sensible plants will be exposed to in this case. This

evaluation helps us to discern if they may significantly influence r the results obtained by the candidate strains (Figure 5).

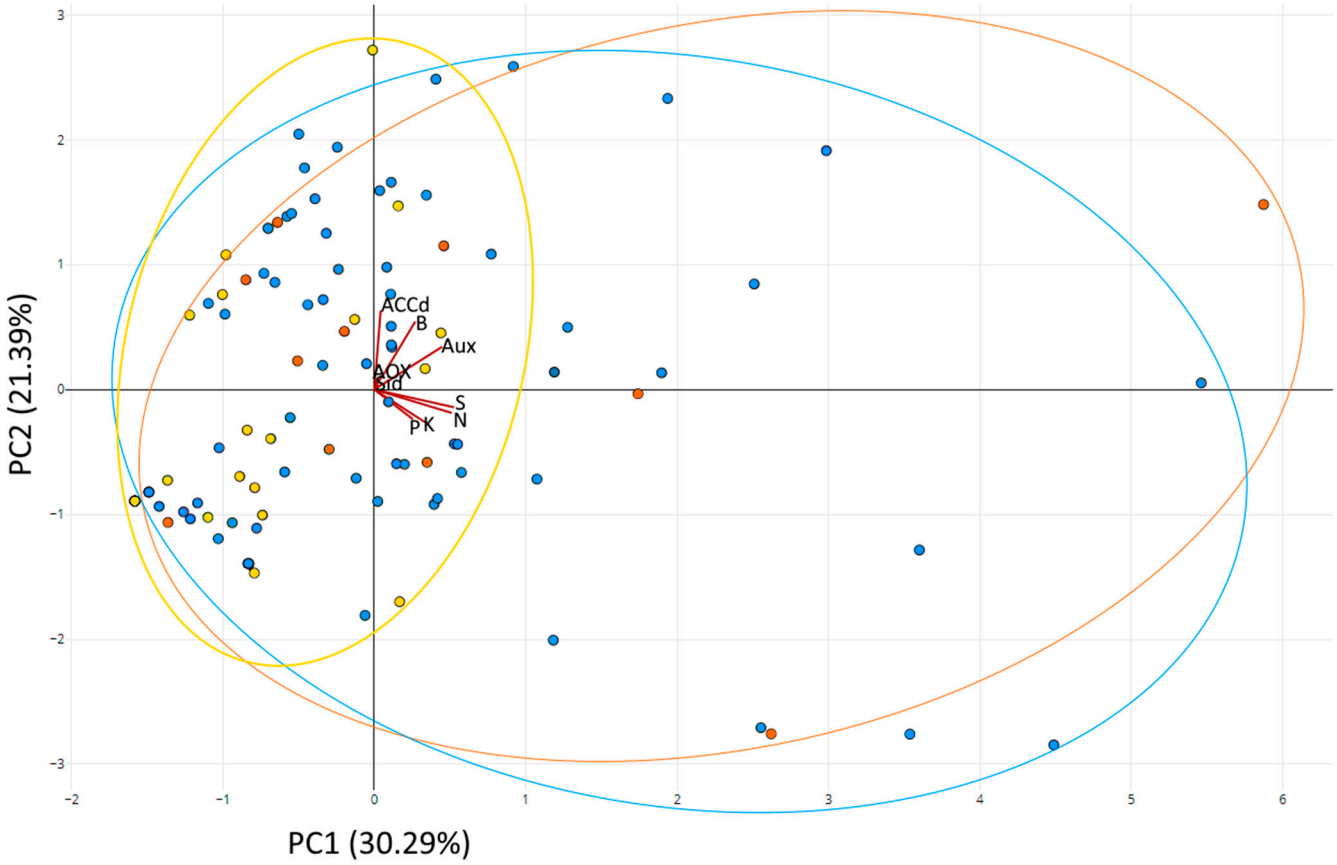

Figure 4. Principal component analysis (PCA). The first two principal components from a principal component analysis using *r*log transformed expression values. Principal component analysis (PCA) of strains isolated only in salt-rich soils (blue), only in gypsum-rich soils (yellow), and in both types of samples (orange). The first principal component (PC1, x-axis) explains 30.29% of the variation in the data, while the second principal component (PC2, y-axis) increases total explained variation to 21.39%. A confidence ellipsis at 99% is drawn for each group. Each trait evaluated was represented with a red vector in the graph. Each trait evaluated was represented with a red vector in the graph were 'N', corresponding to nitrogen fixation; 'P', to phosphorus solubilization; 'K', to potassium solubilization; 'S', to sulfur oxidizing; 'Sid' to siderophores production; 'biofilm', to biofilm production 'ACCd', to the growth in ACC deaminase medium; 'B', to biofilm production; 'Aux', to auxins production; and 'AOx', to antioxidants production.

Thus, starting with the effects of osmotic stress on the growth curve of the candidate strains, we observed that *S. stutzeri* A38 and *P. frigoritolerans*/*simplex* A70 did not show significant differences, whereas the growth of the remaining strains was reduced by approximately 30%. With this in mind, we made adjustments to the subsequent characterization tests. Under stressing conditions, the production of antioxidants was similar or even slightly higher, but not significant enough. In the case of the *S. stutzeri* A38 strain, it was the only strain that reduced antioxidant production by approximately 40%.

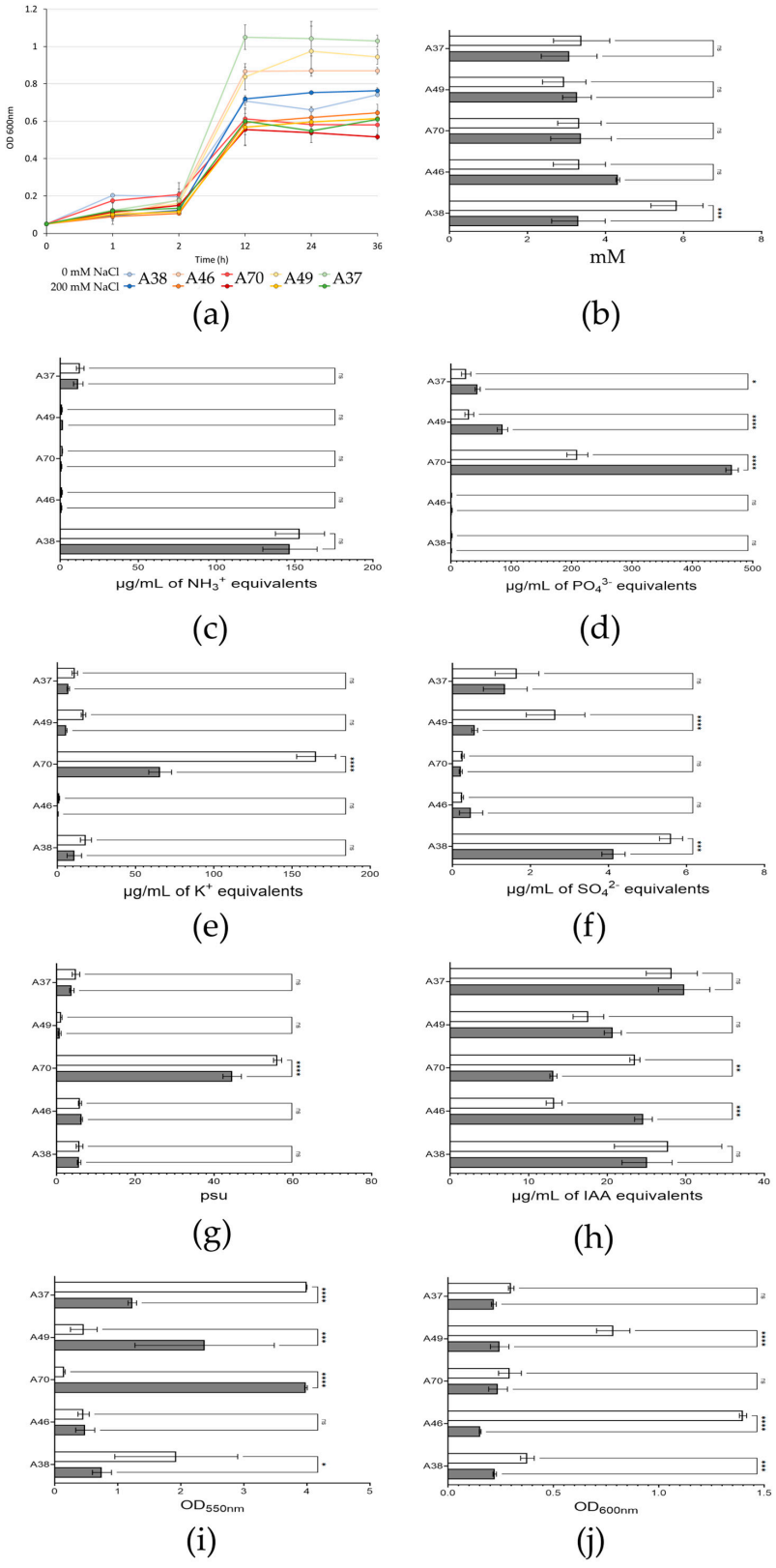

Figure 5. Performance of candidate strains under high osmotic conditions. The line graphs represent the growth curves of the candidate strains in regular conditions, and in osmotic stress caused by supplementation of 200 mM of NaCl in the growth medium (**a**). Likewise, the graph bars represent the performance of the candidate strains under such conditions (white for control, and grey for osmotic stress conditions) for antioxidant-equivalent production in mM (**b**), nitrogen fixation (in NH^{3+}

equivalents) (**c**), phosphate solubilization (in PO_4^{3-} equivalents) (**d**), potassium solubilization (in K^+ equivalents) (**e**), sulfur oxydation (in SO_4^{2-} equivalents) (**f**), production of siderophores (in percent siderophore units, psu) (**g**), auxin production (in indoleacetic (IAA) equivalents) (**h**), biofilm production (in optical density (OD) units) (**i**), and growth in ACC deaminase medium (in OD units) (**j**). Here, the label 'A37′ stands for *N. circulans* A37; 'A49′, for *B. pumilus* A49; 'A70′, for *P. frigoritolerans/simplex* A70; 'A46′, for *B. licheniformis* A46; and 'A38′, for *S. stutzeri* A38. The columns in white represent the control treatments; meanwhile, the grey bars represent the osmotic stressing treatment. The asterisks represent a statistically significant difference at $p < 0.05$ *, $p < 0.01$, ** $p < 0.001$, ***, and $p < 0.0001$, ****; meanwhile, ^{ns} stands for sets with no statistical difference with respect to the control. Error bars represent s.d.

Regarding nitrogen fixation, strain *S. stutzeri* A38 produced 153 µg/mL NH_3^+ equivalents, and no significant change was detected when osmotic stress was included. For phosphate solubilization, strains *B. licheniformis* A46 and *S. stutzeri* A38 did not show any change, whereas strains A37, *B. pumilus* A49, and *P. frigoritolerans/simplex* A70 increased solubilization more than twice when osmotic stress was included. Strain *P. frigoritolerans/simplex* A70 was able to solubilize up to 466 µg/mL of PO_4^{3-} equivalents. For potassium solubilization, no significant change was detected, except for strain *P. frigoritolerans/simplex* A70 (166 µg/mL of K^+ equivalents), which decreased by approximately 2.5 times. When sulfur oxidation was evaluated, most strains obtained very similar or slightly higher values, but with no significant difference. Here, strains *S. stutzeri* A38 (5.61 µg/mL of SO_4^{2-} equivalents) and *B. pumilus* A49 (2.65 µg/mL of SO_4^{2-} equivalents) saw their values reduced by 25% and 80%, respectively, when osmotic stress conditions were included. Siderophore production was not affected by stressful conditions, except in strain *P. frigoritolerans/simplex* A70 (56.14 psu), where production was reduced by approximately 20%.

In the production of auxins, the *N. circulans* A37, *S. stutzeri* A38, and *B. pumilus* A49 strains maintained their production level under stress, but in the *P. frigoritolerans/simplex* A70 strain, it was reduced by 40%, while for *B. licheniformis* A46, this decrease even reached 2.5 times. Interestingly, the production of strain *B. licheniformis* A46 almost doubled under osmotic stress. Biofilm production was reduced for strains *S. stutzeri* A38 and *N. circulans* A37 more than 2- and 3-fold, respectively; however, for the *B. pumilus* A49 strain, biofilm production was multiplied by more than 26 times under stress conditions. Here, the *B. licheniformis* A46 strain did not undergo any significant changes. Finally, growth with ACC as the sole source of C and N, was not affected for the strains *N. circulans* A37 and *P. frigoritolerans/simplex* A70. However, this was limited when osmotic stress was included for strains *S. stutzeri* A38, *B. pumilus* A49, and *B. licheniformis* A46, falling by 40%, 3, and 10 times, respectively.

3.4. Evaluation of In Vitro Colonization and Germination Traits under Osmotic Stress Conditions

The colonization ratio of the candidate strains was evaluated in vitro under regular and osmotic-stressing conditions in *Medicago sativa*, *M. polymorpha*, and *M. littoralis* (Figure 6a–c). Moreover, their effects over the germination ratio were also assessed (Figure 6a,b). Starting with germination tests on *M. sativa*, we verified its high sensitivity to osmotic stress, as it was not able to germinate under these conditions. Regarding the treatments with bacterial solutions, germination increased when the seeds were treated with the strains *B. pumilus* A49 (15%), *B. licheniformis* A46 (20%), and *N. circulans* A37 (25%), whereas it was maintained or slightly decreased with *P. frigoritolerans/simplex* A70 and *S. stutzeri* A38. With osmotic stress treatment, all inoculants were capable of inducing germination, which was reduced by 25–35% compared to non-stressful conditions. Strain *S. stutzeri* A38 maintained similar levels of germination under both conditions.

In *M. polymorpha*, germination under control conditions reached 90%, reducing by only 30% due to the effect of osmotic stress. The bacterial treatments maintained similar levels of germination in the cases of *P. frigoritolerans/simplex* A70 and *B. licheniformis* A46, but germination fell between 25 and 50% when the seeds were treated with *N. circulans*

A37, *B. pumilus* A49, or *S. stutzeri* A38. When osmotic stress was applied, the germination ratio was maintained for *N. circulans* A37, *P. frigoritolerans/simplex* A70, and A38, whereas it decreased between 25 and 50% for the treatments with *B. pumilus* A49 and *B. licheniformis* A46.

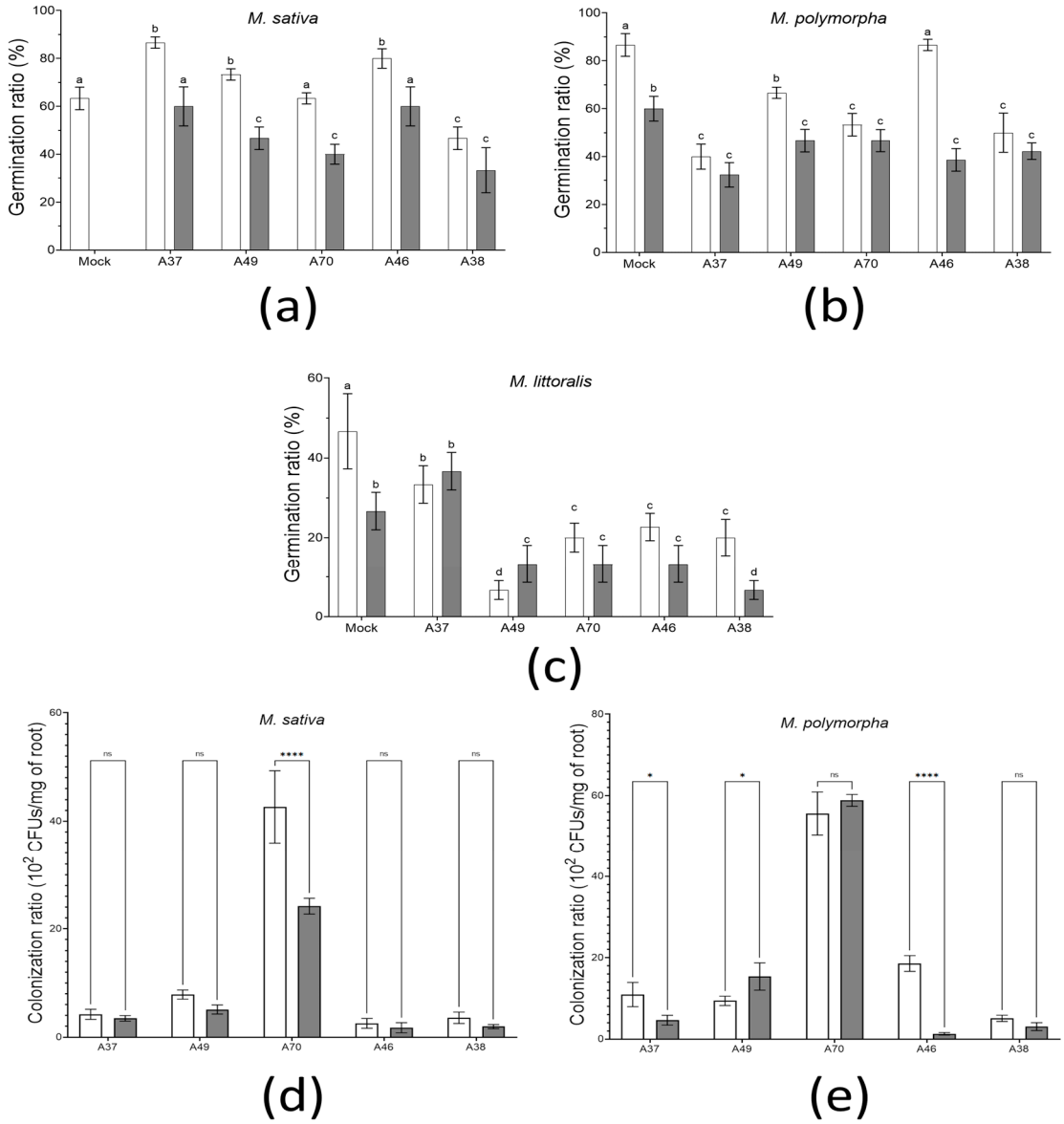

Figure 6. Germination and in vitro colonization ratio. The graph bars represent the germination ratio recorded after treatment with the candidate strains in M. sativa (**a**), *M. polymorpha* (**b**) and *M. littoralis* (**c**), and the colonization ratio showed by the candidate strains in *M. sativa* (**d**) and *M. polymorpha* (**e**). Here, the label 'Mock' stands for mock treatment; 'A37', for *N. circulans* A37; 'A49', for *B. pumilus* A49; 'A70', for *P. frigoritolerans/simplex* A70; 'A46', for *B. licheniformis* A46; and 'A38', for *S. stutzeri* A38. The columns in white represent the control treatments, meanwhile the grey bars represent the osmotic stressing treatment. The sets of data (n = 25) were compared using a two-ways ANOVA, where the letters indicate same significance level; alternatively, the asterisks represent a statistically significant difference at $p < 0.05$ *, and $p < 0.0001$, ****; meanwhile ^{ns} stands for sets with no statistical difference respect to the control. Error bars represent s.d.

Finally, in *M. littoralis*, germination was approximately 50% under non-stressful conditions, which decreased to 40% when saline stress was applied. When they were treated with *N. circulans* A37, germination was reduced by 25%, while with *P. frigoritolerans/simplex*

A70, *B. pumilus* A49, and *S. stutzeri* A38, it was reduced by almost half. However, when osmotic stress was applied, *P. frigoritolerans/simplex* A70 and *B. pumilus* A49 did not lose their germination rate, and the treatment with *S. stutzeri* A38 germinated almost twice as much. Particularly interesting is the case of the treatment with *B. pumilus* A49, whose germination fell by 85% under regular conditions but increased to almost double when the seeds were exposed to osmotic stress.

In general, the colonization ratios observed for the candidate strains were similar in both plant species (around 0.5–2.0 $\times$ 10^3 CFU/mg of root), except in the case of the candidate strain *P. frigoritolerans/simplex* A70, which doubled and almost tripled the colonization ratios of other strains in *M. sativa* (4.2 $\times$ 10^3 CFU/mg of root) and *M. polymorpha* (5.6 $\times$ 10^3 CFU/mg of root), respectively, under regular experimental conditions. However, when osmotic stress conditions were applied in *M. sativa*, this was the only strain that significantly lost the colonization rate (43%). Interestingly, this did not occur in *M. polymorpha*, where no significant difference was observed in the colonization ratio. In addition, strain *S. stutzeri* A38 did not lose colonization rate when osmotic stress was applied, but strains *N. circulans* A37 and *B. licheniformis* A46 did it by 58% and 93%, respectively. Strain *B. pumilus* A49 was the only strain that increased its colonization rate, achieving 60% more. Unfortunately, evaluation of the colonization of the candidate strains in *M. littoralis* could not be carried out, as the radicles of the seedlings of this species did not reach the minimum size required for the test in the growth medium.

3.5. Biotreatment Tests

After the application of the candidate strains, each one assuming an alternative strategy or treatment, an osmotic stress was applied adjusting the irrigation to 200 mM with NaCl. This treatment had a visible effect on different sets of plants, as shown in Figure 7. Here, we recorded a clearer impact on root length than on plant height, both in *Medicago sativa* and *Medicago polymorpha*. Moreover, they showed a better root architecture (secondary roots, tertiary roots, and hairy roots); however, many radicles in both plant species were fragile and often broke during preparation, so we decided not to include this analysis to avoid biased results. Quantification of the parameters, length of the main root, height of the shoot, and total biomass (full-plant dry weight) allowed us to emphasize some of the main effects caused by the different treatments (Figure 8). Starting with the root of *M. sativa* (Figure 8a), we found that in the condition without bacterial treatment (mock), it reached approximately 11 cm, falling to practically half under osmotic stress conditions. Although no treatment with the candidate strains resulted in a larger average root size, some treatments showed remarkable effects. Thus, the seedlings treated with *B. licheniformis* A46 and *N. circulans* A37 showed shorter roots under osmotic stress conditions (15 and 39%, respectively), whereas those treated with *P. frigoritolerans/simplex* A70 maintained the same level under both conditions. In contrast, the seedlings treated with *B. pumilus* A49 and *S. stutzeri* A38 increased the average size of their roots between 46 and 57%. In the case of *M. polymorpha* (Figure 8b), the mock set (approximately 12 cm) lost only 30% of the average length of its roots when osmotic stress was applied. Here, the *N. circulans* A37 treatment showed a similar pattern to the mock set as well as after the *P. frigoritolerans/simplex* A70 treatment, but this set of plants did not lose root length when osmotic stress was applied. However, the seedlings treated with *B. pumilus* A49 were able to grow about a 57% more under conditions of osmotic stress, similarly as with the treatment of *B. licheniformis* A46 and *S. stutzeri* A38.

Analyzing the height of the shoot, the *M. sativa* seedlings of the mock set (approximately 5.5 cm) also lost slightly more than 50% of the value, as was already recorded in the root length (Figure 8c). In general, no treatment showed a significant difference, except in the set treated with *S. stutzeri* A38, where the average size of the seedlings increased by approximately 26%, losing only 17% of this value when stress conditions were applied. In *M. polymorpha* seedlings (Figure 8d), no significant difference was found between the bacterial treatments and the height reached by the mock set (approximately 4.2 cm); moreover,

the application of osmotic stress did not seem to affect the height reached by any of the sets analyzed.

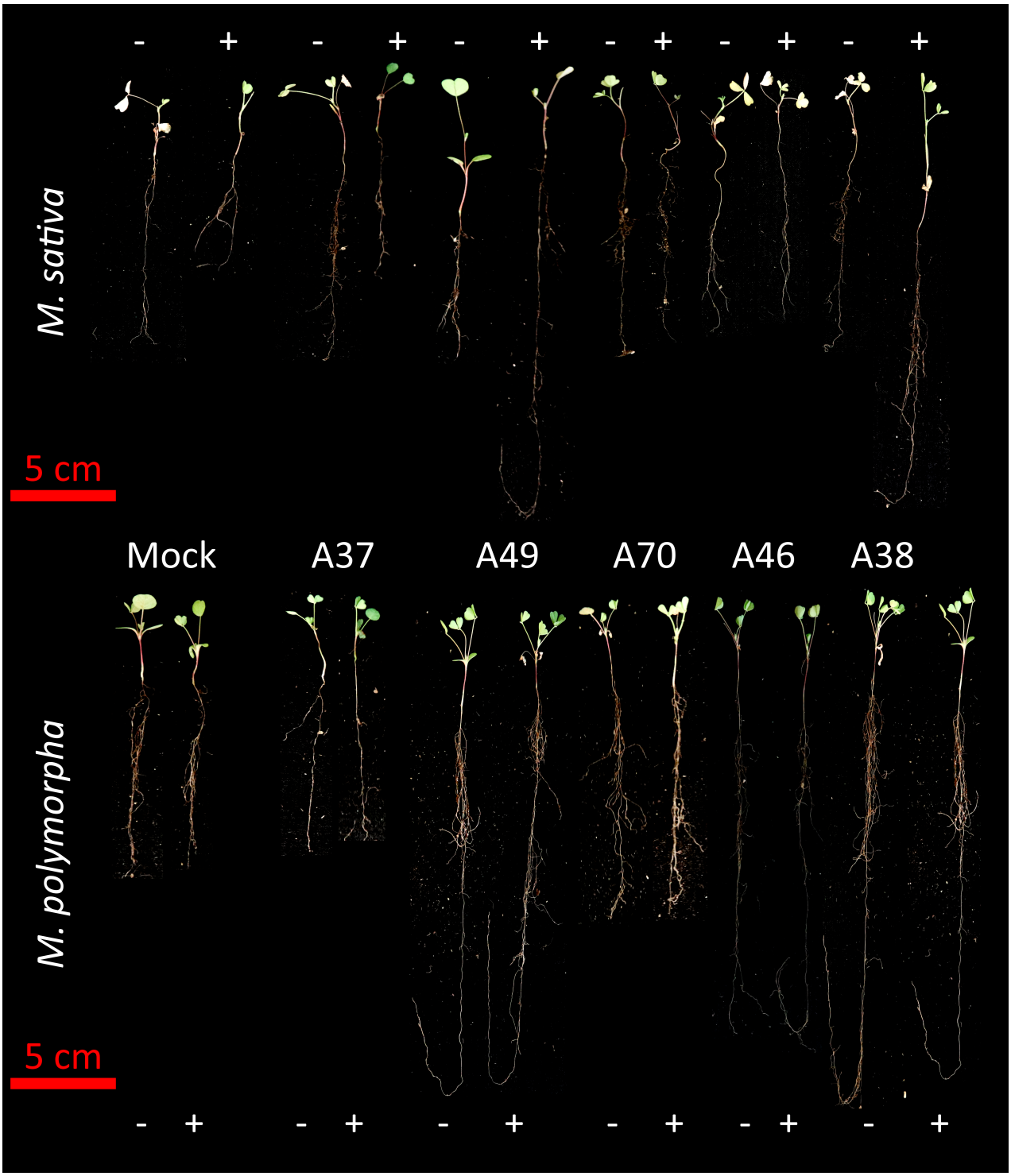

Figure 7. Phenotype evaluation in full plants. The full plant pictures show the representative phenotype recorded in *M. sativa* and *M. polymorpha* after treatment with the candidate strains. Here, the label 'Mock' stands for mock treatment; 'A37', for *N. circulans* A37; 'A49', for *B. pumilus* A49; 'A70', for *P. frigoritolerans*/*simplex* A70; 'A46', for *B. licheniformis* A46; and 'A38', for *S. stutzeri* A38. Moreover, the labels '-' indicate control condition without osmotic stress, and '+', condition with osmotic stress.

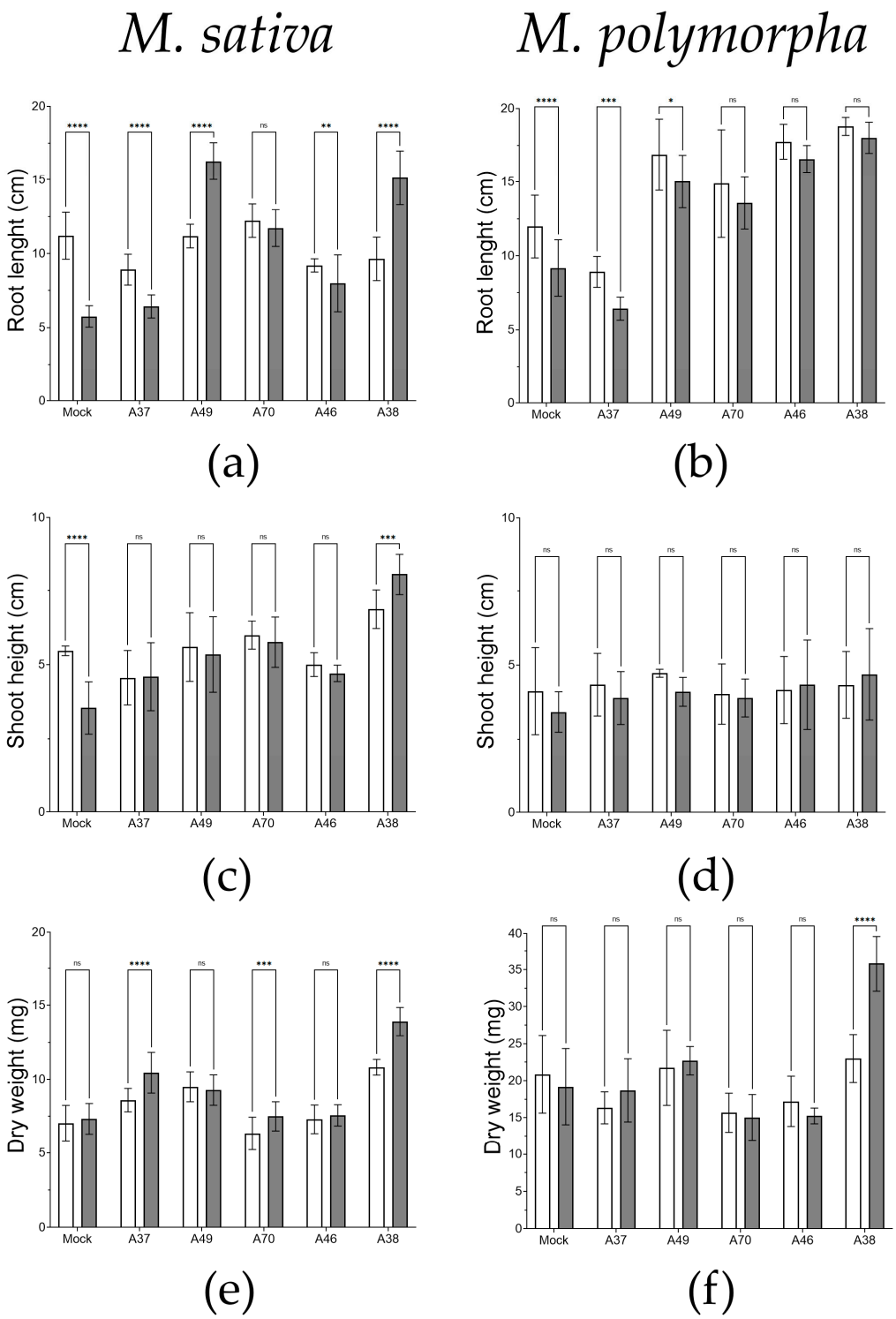

Figure 8. Phenotyping quantification. The graph bars represent the root length (**a**,**b**), shoot height (**c**,**d**), and full-plant dry weight biomass (**e**,**f**) recorded in *M. sativa* and *M. polymorpha* after treatment with the candidate strains. Here, the label 'Mock' stands for mock treatment; 'A37', for *N. circulans* A37; 'A49', for *B. pumilus* A49; 'A70', for *P. frigoritolerans*/simplex A70; 'A46', for *B. licheniformis* A46; and 'A38', for *S. stutzeri* A38. The columns in white represent the control treatments; meanwhile, the grey bars represent the osmotic stressing treatment. The sets of data (n = 25) were compared using a two-way ANOVA, where the asterisks represent a statistically significant difference at $p < 0.05$ *, $p < 0.01$, **, $p < 0.001$ ***, and $p < 0.0001$, ****; meanwhile, ^{ns} stands for sets with no statistical difference with respect to the control. Error bars represent s.d.

Finally, the total biomass reached in the control seedlings of *M. sativa* (approximately 7 mg dry weight) was maintained under stress conditions (Figure 8e). When the seedlings were treated with *B. pumilus* A49 or *S. stutzeri* A38, their weight increased by 35 and 54%, respectively. Under stress conditions, the dry weight of the sets treated with *P. frigoritolerans/simplex* A70, *N. circulans* A37, and *S. stutzeri* A38 increased by 18, 21%, and 30%, respectively. Following this, the *M. polymorpha* mock set (21 mg of dry weight) did not lose biomass when stressful conditions were applied (Figure 8f). Regarding the rest of the treatments, it is worth noting that the weight of seedlings treated with *S. stutzeri* A38 increased by approximately 56%. The rest of the conditions did not show significant differences.

4. Discussion

Most projections for the Mediterranean region, as well as many subtropical areas, anticipate a substantial decrease in precipitation levels coupled with a notable rise in average temperatures. This will lead to an overall increase in evapotranspiration rates. These climatic shifts can expedite processes such as desertification, soil salinization, gypsum concentration, and consequently, increase in the osmotic stress for plants [68,69]. The latter phenomena may be further exacerbated by a sea level rise. Already, a significant portion (18%) of growing areas in the Iberian Peninsula is under threat from these advancing conditions, casting doubt on the region's future agricultural production capacity [70,71]. Furthermore, numerous farmlands are either overexploited, depleted, or abandoned due to their high ionic content. This suggests that their prospects for use or reclamation are dimming in the face of the progressing conditions mentioned above [71]. Consequently, agriculture confronts the imperative of identifying more sustainable management strategies that optimize water use and potentially rejuvenate unproductive land. This is crucial to meet the demands of a growing human population. Our proposition is rooted in the observation that within environments characterized by high ionic content, we can identify microorganisms best suited for enhancing crop yields under osmotic stress conditions. To evaluate this premise, we isolated bacteria from salt pans, salt flats, and soils with substantial gypsum accumulation, irrespective of their prior involvement in agricultural activities. Consequently, in addition to discovering highly adapted species from saline environments (such as *Bacillus subtilis* or *Priestia megaterium*), we encountered several commonplace species from other challenging environments (including *Bacillus mojavensis*, *Bacillus thuringiensis*, or *Pantoea agglomerans*). Furthermore, we identified both less common and more prevalent species from non-stressful environments, such as *Niallia nealsonii* or *Stutzerimonas stutzeri* [72–76]. The wide-ranging distribution of many of these species is noteworthy. Despite possessing mechanisms akin to those exhibited in non-stressful environments, these strains evolved to thrive in and adapt to these challenging habitats. This offers us a broader and more promising array of options for advancing future treatments. Considering our own samples, we conclude that, due to the amount recovered and the reproducibility, they can be a good representation of each environment. In this sense, about 12% and 65% of the strains were uniquely happening in gypsum-rich and salt-rich soils, respectively. The selection process focused our studies in salt-originated strains, probably showing how these environments are more restrictive in terms of osmotic stress.

One of our primary concerns in characterizing and assessing the most promising candidates for various strategies was validating their capabilities under stressful conditions. While most studies conduct in vitro screenings to efficiently gauge the general capacities of each strain, this approach does not guarantee similar effects on plants, especially in stressful conditions [72–74,77]. Therefore, we opted to incorporate osmotic stress into different trials as a distinguishing factor, aligning with a contextualizing trend emerging in the field [75,76].

This allowed us to ascertain whether the selected candidate strains could maintain, or even enhance, their performance levels observed in the selection trials conducted under regular conditions. In instances where results diminished, we gained insight into the

anticipated level of impact. Additionally, the inclusion of plant-controlled trials ensured compatibility with prospective treatments [64]. It is noteworthy that none of the strains isolated from hypergypsic environments were chosen. This decision was based on the challenge of accurate identification, and for safety precautions, we opted not to proceed with them. Nevertheless, certain strains from this origin demonstrated sufficient promise to warrant further characterization and application studies in the future [78].

The selected strains demonstrated exceptional performance in one or more of the selection trials. Previous studies indicated that the production of antioxidants can mitigate osmotic stress [79,80]. With this in mind, we opted for the *Bacillus pumilus* A49 strain, which notably excelled in enhancing the root development of *M. sativa* and *M. polymorpha*. This effect was even more pronounced under conditions of osmotic stress in *M. sativa*. This improvement may be attributed to the regulation of reactive oxygen species (ROS) production within the plant, a process that is significantly heightened during osmotic stress. By modulating ROS levels, better root development is facilitated. Earlier reports also highlighted how antioxidants can foster and induce root development by alleviating the pressure of ROS generated during this process [81]. *Bacillus pumilus* A49 is renowned for its robust tolerance to stressful environments and was suggested as a potential treatment against osmotic stress [82,83]. In fact, Kumar and colleagues specifically noted that soils inoculated with *Bacillus pumilus* bacteria can stimulate plant growth, augment soil microbial counts, and enhance soil enzyme activity [82].

We also chose *Niallia circulans* A37 due to its notable biofilm formation and auxin production. The biofilms produced by many strains were recognized for their protective role against ionic toxicity and in alleviating osmotic pressure on roots [84,85]. Additionally, the auxin production by this strain can enhance the root architecture of treated plants under osmotic stress [34]. However, upon evaluating its effects, we observed only a slight improvement in the biomass of *M. sativa* under osmotic stress conditions. Although this outcome deviated from our initial expectations, it was evident that the treatment mitigated the loss of root length. *Niallia circulans* strain is acknowledged for its high auxin production and its role as a plant growth promoter [86,87], though it is not typically classified as a salt-protecting strain. Another intriguing strategy hinges on regulating ethylene levels during osmotic stress through the production of ACC deaminase [73,88]. Consequently, treatment with *Bacillus licheniformis* A46 strain led to enhanced root growth in *M. polymorpha* seedlings, maintaining similar levels even under osmotic stress conditions. In other parameters, we noted that this treatment effectively stabilized the metrics, potentially diluting any adverse effects induced by osmotic stress. While these results may not be highly representative, they are significant because they indicate that the plant can more effectively regulate stress and sustain its development. While this enzyme demonstrated growth-improving effects in other instances, numerous studies showed that its impact on ethylene production in plants is balanced under stress conditions [73,89]. This suggests that the plant is less stressed and better able to maintain its normal development. *Bacillus licheniformis* strain was previously identified as halotolerant and capable of promoting plant growth [90]. It was found to notably enhance germination rate, root length, and seedling dry weight by utilizing ACC as a nitrogen source, even under varying levels of salt osmotic stress [91].

Lastly, for strategies focusing on nutrient accessibility—an essential factor compromised under conditions of osmotic stress—we selected two strains: *Stutzerimonas stutzeri* A38 as the nitrogen fixator and sulfur oxidizer, and *P. frigoritolerans/simplex* A70 as the solubilizer of phosphorus (P) and potassium (K), as well as a producer of siderophores. In the case of *Stutzerimonas stutzeri* A38, known for its nitrogen-fixing abilities and as a biocontroller strain, treatment with this strain prompted notable root system growth in both plant species [92,93]. Both strains *P. frigoritolerans* and *P. simplex* were recognized as nematicides and biocontrollers, with some studies suggesting their potential as stress-alleviating strains [94,95]. When seedlings were treated with *P. frigoritolerans/simplex* A70, the most notable effect was the maintenance of values akin to those obtained under regular conditions even when osmotic stress was applied. Seedlings treated with this strain did

not experience the negative impacts observed in the control sets. This could be attributed to the fact that, as indicated in other studies, enhanced accessibility to phosphorus and potassium can improve salt tolerance [96–98]. In summary, it is evident that all of these diverse approaches have the potential to enhance crop performance to some degree under osmotic stress conditions. The obtained results are particularly noteworthy in that all candidate strains significantly improved the germination rate of *M. sativa* under osmotic stress conditions [99,100]. Overcoming the challenge of germinating many genotypes of this species under stress is a pivotal factor in direct field sowing [100]. Previous studies demonstrated that certain inoculants can enhance this aspect, increasing the likelihood of successful development and future production [101,102]. Although *M. polymorpha* and *M. littoralis* exhibited lower sensitivity to salinity (albeit highly dependent on the genotype [29,103]), they also benefited from the application of strains *P. frigoritolerans/simplex* A70, *Stutzerimonas stutzeri* A38, and *Niallia circulans* A37, which led to improvements in their performance. However, in specific cases, such as *P. frigoritolerans/simplex* A70 for *M. sativa*, treatments may lead to a decrease in this ratio. Thus, it is imperative to conduct case-specific studies to prevent unintended effects. On a different note, all the treatments exhibited similar or, in many cases, higher values compared to the control group, both under normal conditions and in the presence of osmotic stress. This underscores that the selection process for growth promotion was further reinforced by evaluating these traits under osmotic stress conditions. Notably, strains *Bacillus pumilus* A49 and *Bacillus licheniformis* A46 demonstrated significant effects on the root system development of both *M. sativa* and *M. polymorpha*. While root development in many plant species is typically constrained under stress conditions, the influence of root-associated bacteria can enhance this process, helping to alleviate osmotic pressure and access less readily available water sources [41,104]. Furthermore, *Stutzerimonas stutzeri* A38 emerged as the most effective strain for biotreatment, showing improvements across all parameters under osmotic stress conditions in both plant species. Its results are more akin to those observed in treatments involving well-established bacteria known for alleviating osmotic stress in plants. This indicates a particularly promising potential for *Stutzerimonas stutzeri* A38 as a beneficial biotreatment [73,80,105,106].

Addressing the challenges posed by salinization demands a sustainable and safe alternative. We believe that this localized approach, utilizing regional resources, could be a valuable addition to the formulation of new inoculants that are better integrated into the environment and equipped to deploy diverse strategies [107]. Given the intricate nature of osmotic stress, it calls for approaches that are more nuanced than those solely focused on promoting growth. Some researchers started to recognize the necessity of enhancing the applicability and coherence of inoculants in soils and climates specific to affected regions [85,106–108]. However, further studies are needed in this area to facilitate the development of tailor-made tools. These might encompass the utilization of combined strategies (such as SynComs, communities, or synthetic consortia) or examining the critical periods for applying treatments that alleviate osmotic stress. Contextualizing new-generation inoculants within their ecological settings is an imperative step to enhance the efficacy of biotreatments in the future.

5. Conclusions

Salinization processes are exacerbating, leading to significant yield losses across various crops. This underscores the critical importance of delineating new treatments to enhance resilience to osmotic stress. While numerous studies advocate for the efficacy of diverse bacterial strains as potential treatments, our research emphasizes the imperative of establishing stringent performance criteria under stress conditions for the selection of novel inoculants. To this end, we integrated in vitro assays for growth promotion and osmotic stress mitigation, alongside assessments of crucial plant traits such as germination and colonization. This established a baseline quality threshold for progression to large-scale trials. Subsequently, we observed varying degrees of impact on different physiological

processes and plant responses, discerning clear patterns of effect across different strategies. Notably, strains A38 (selected for nitrogen fixation and sulfur oxidation), A46 (chosen for ACC deaminase production), and A49 (identified as an antioxidant producer) emerged as the most promising candidates, exhibiting marked improvements in overall growth, root development, as well as germination and colonization traits. Future trials may encompass the formulation of consortia-comprising strains employing different strategies, long-term assessments, competition with local microfauna, and evaluations of productivity in diverse field crops to validate the potential of these promising candidates.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/soilsystems7040086/s1, Figure S1: Petri dish-based screening methods.

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