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THE EFFECT OF DIFFERENT TREATMENT DURATIONS WITH INDOLE-3-ACETIC ACID (IAA)-PRODUCING ENDOPHYTIC *BACILLUS* SPP. ON TOMATO 'AICHA' UNDER SALINE CONDITIONS

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ABSTRACT

This study aimed to investigate the effect of indole-3-acetic acid (IAA)-producing endophytes from saline environments on survival and growth of tomato under salt stress, using different treatment times. Twenty-five endophytic bacteria were isolated from halophytes (*Atriplex halimus* L., *Tamarix aphylla* L.) issued from saline regions (Algeria). Five strains were selected based on tolerance to NaCl. A polyphasic taxonomic study was conducted. The selected strains were assigned to the Bacillus cereus group. Quantification of IAA production was done by high-performance liquid chromatography (HPLC). An *in vitro* test of growth promotion/protection of tomato 'Aicha' was conducted under saline conditions (50 mM, 75 mM, 100 mM, and 125 mM), for different durations of seed biopriming (1h, 3h, and 12h). Endophytes led to a significant amelioration of salt stress in tomato 'Aicha' at all salinity levels, depending on treatment times, indicating the necessity of optimizing this crucial factor.

Keywords: endophytes, *Bacillus*, indole-3-acetic acid (IAA), treatment durations, salt stress, tomato

INTRODUCTION

Tomato crops are considered as some of the most important ones in the world **(Flores *et al.*, 2010)**. Fertilizers and water are supplied extensively, particularly under protected cultivation, for maximal production, resulting in soil degradations, mainly linked with the accumulation of phosphate, nitrate, and salinity **(Shi *et al.*, 2009)**. In hydroponic systems, salinity results from the continuing recirculation of drain solution and the salinity of irrigation water **(Carmassi *et al.*, 2005)**. This abiotic stress delays tomato seed germination and plant growth, thus, causing yield losses **(Cuartero and Fernández-Muñoz 1999)**. Salinity resistance/tolerance improvement of tomato by breeding programs has been limited due to its physiological and genetic complexity **(Cuartero *et al.*, 2006)**. The development of novel strategies to increase salinity tolerance is a principal objective to overcome salinity stress on tomato **(Asins *et al.*, 2010)**.
Endophytic plant growth-promoting bacteria (PGPB) have the ability to colonize a plant's inner tissues and to establish a special type of relationship where both partners may obtain benefits from this interaction **(Hallmann *et al.*, 1997)**. Plant growth is directly promoted by PGPB either by facilitating nutrition uptake or by modulating the plant hormone levels. Indirect promotion of plant growth occurs via PGPB, decreasing the inhibitory effects of various pathogenic agents on plant growth and development (i.e., acting as a biocontrol agent) **(Yekkour et *al.*, 2012; Goudjal *et al.*, 2014; Toumatia *et al.*, 2016)**.
Moreover, inoculation of seeds with beneficial microorganisms in combination with priming (biopriming) can promote rapid and more uniform seed germination and plant growth, improve the versatility of crop performance and stabilize the efficacy of biological agents in the present fragile agricultural ecosystem by reducing dependency on chemical inputs **(Sharma *et al.*, 2015)** and by ameliorating a wide variety of biotic, abiotic and physiological stresses to seed and seedlings **(Rajendra Prasad *et al.*, 2017).**
This study aimed to investigate the effect of treatment duration using endophytic bacteria from harsh saline environments on tomato survival and growth under salt stress conditions.

MATERIAL AND METHODS

Isolation of root endophytic bacteria

This study aimed to investigate the effect of treatment duration using endophytic bacteria from harsh saline environments on tomato survival and growth under salt stress conditions. The halophytes *Atriplex halimus* L. (Amaranthaceae) and *Tamarix aphylla* L. (Tamaricaceae) were selected for root sampling (at least 10 cm from the soil surface; EC= 24 mS/cm) from two regions, S1 (coastal sebkha of Daia, region of Oran, 35°36'09.1"N, 0°26'13.7"W) and S2 (continental sebkha of El Mosrane locality, region of Djelfa, 34°56'08.0"N, 3°03'19.8"E). It is worth noting that both soils belong to the SET#7 according to the FAO World Reference Base (WRB) classification **(IUSS Working Group WRB, 2015)**. The roots were then placed in sterile containers and stored in a dry cool place (4 °C).
They were subsequently cleaned with tap water and cut into 4-5 cm long strands. Root surface sterilization was realized by soaking each root strand in oxygenated water (10 v) for 15 s. They were then rinsed three times with Mg SO_4 0.1 M (Sigma-Aldrich, Darmstadt, Germany), to remove H_2O_2 residual. The endophytic microflora was released in 60 mL sterile tap water by vigorous grinding of the surface-sterilized root strands for 3-5 min in an aseptic porcelain mortar. The resulting turbid suspension was divided into two test tubes (18 mL), each used for inoculating enrichment tubes. To increase the initial biomass, aliquots of 100 µL of homogenate were inoculated into 10 mL of nutrient broth (Fluka, Buchs, Switzerland) and incubated for 24-36 h at 30 °C **(Bashan *et al.*, 1993)**.
The isolation was performed on trypticase soy agar (TSA) medium (Fluka, Buchs, Switzerland) diluted to 1/10 in order to recover a maximum of microbial diversity. To select the endophytic halotolerant microflora, the TSA medium was slightly modified (saline 1/10 TSA) by adding NaCl (Sigma-Aldrich, Darmstadt, Germany) to a final concentration equivalent to the salinity of the soil samples (1.3 %) (from each enrichment tube at least 10 plates of the selected isolation media were streaked for the isolation of endophytic bacteria). A volume of 100 µL was deposited and spread on saline 1/10 TSA using a loop. The incubation was carried out at 30 °C for 24 h. Microbial colonies were selected (predominant macromorphologies) for isolation. The resulting strains were stored in saline TSA broth (1.3 % NaCl) (Fluka, Buchs, Switzerland) with 20 % glycerol (Sigma-Aldrich, Darmstadt, Germany) at - 20 °C **(Bashan *et al.*, 1993)**.

Salt tolerance screening

Twenty-five bacterial strains with diverse morphologies have been tested for their resistance to different concentrations of NaCl on TSA. After a pre-culture, each isolate was sub-cultured on TSA with final NaCl concentrations of 0.5, 1, 2, 2.5, 3.5, and 5 %. After 24-48 hour incubation, results were recorded. At the end of this test, non-redundant representatives of different morphotypes, showing resistance to a wide range of NaCl concentrations were selected for the identification and the plant test.

Bacterial identification

The selected isolates were subjected to a phenotypic identification based on the macromorphology of bacterial colonies on TSA (colony color, size, opacity,

pigment distribution, and growth), in addition to the micromorphology using Gram staining (cell shape and size; endospore: presence/absence, type, size, location; arrangement mode and motility). Furthermore, a catalase test was performed in addition to the determination of the respiratory type on the meat-liver agar. In addition to these conventional characteristics, biochemical identification by API System (20E) (bioMérieux, Marcy l'Etoile, France) was conducted. The micro-galleries were prepared and inoculated with the selected strains and read according to the manufacturer's instructions.

For the 16S rRNA gene sequence analysis, strains were grown at 30 °C for 24 h under stirring (250 rpm) in a 500 mL Erlenmeyer containing 100 mL of trypticase soy broth (TSB) (Fluka, Buchs, Switzerland). The genomic DNA was extracted by the method of **Liu *et al.* (2011)**. The 16S rRNA gene was amplified by PCR using an Invitrogen kit and two primers; 10-30F (5'-GAGTTTGGCGATCCTTCA-3') and 1500R (5'-AGAAAGGTGGAGATCCAGCC-3') (Thermo Fischer Scientific, MA, USA) **(Rainey *et al.*, 1996)**. The final volume of 50 µL of reaction mixture contained 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9.0 at 25 °C) (Thermo Fischer Scientific, MA, USA), 1.5 mM $MgCl_2$ (Thermo Fischer Scientific, MA, USA), 200 µM of each dNTP (Thermo Fischer Scientific, MA, USA), 1 pM of each primer (Thermo Fischer Scientific, MA, USA), 1.25 U of Taq DNA polymerase (Thermo Fischer Scientific, MA, USA) and 1 pi (500 ng) of purified DNA. PCR amplification of the 16S rRNA gene was carried out on a Stratagene RoboCycler Gradient 96 thermocycler. Thermal cycling conditions were as follows: denaturation of the target DNA at 98 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, primer annealing at 52 °C for 1 min and primer extension at 72 °C for 2 min. At the end of the amplification process, the reaction mixture was maintained at 72 °C for 10 min and then cooled to 4 °C. The PCR product was detected by electrophoresis on agarose gel (Sigma-Aldrich, Darmstadt, Germany), using ethidium bromide (Sigma-Aldrich, Darmstadt, Germany). The sequencing was performed by the Coulter Genomics company (Paris, France) using the same primers described above. The 16S rRNA gene sequences of all the strains have been deposited in the GenBank database (c.f. *Results*, *Bacterial identification*).

The identification of the phylogenetic neighbors was performed by the BLASTN program **(Zhang *et al.*, 2000)** against 16S ribosomal RNA sequences (Bacteria and Archaea) database, targeting sequences from type material only **(Agarwala *et al.*, 2018)**. Sequences with the highest scores (E-values) were selected for the phylogenetic analysis using MEGA 7 package **(Kumar *et al.*, 2016)**. The 16S rRNA gene sequences of each strain were aligned using MUSCLE **(Edgar, 2004)** with corresponding nucleotide sequences to the closest taxa recovered from the BLASTn results. The evolutionary distances were computed using the Maximum Composite Likelihood method **(Tamura *et al.*, 2004)** and the evolutionary history was inferred using the Minimum Evolution method **(Rzhetsky & Nei, 1992).** The topology of the tree was assessed through the bootstrap analysis **(Felsenstein, 2005)** using 100 re-sampling. The final tree figure was processed using Adobe Illustrator CC 18.1.1 **(Adobe Inc., 2014)**.

IAA quantification by HPLC

Isolates were grown on TSB medium (100 mL) for 48h at 30 °C and protected from light, after which each culture was subjected to centrifugation at 6,000 rpm for 30 min, to recover supernatant. The latter, after acidification to pH 2.5 - 3.0 with 1N HCl (Sigma-Aldrich, Darmstadt, Germany), was extracted by ethyl acetate 1:2 v/v (Sigma-Aldrich, Darmstadt, Germany). The recovered organic phase was dried through a filter paper containing anhydrous sodium sulfate (Sigma-Aldrich, Darmstadt, Germany) to restrain residual traces of water and hydrophilic contaminants. The obtained extract was concentrated to dryness. Each dry extract was re-suspended in methanol. The HPLC quantification of IAA was performed in a Jasco® plus system (Japan) equipped with an Interchim reverse-phase C-18 column (15 µm particle size; 7.8 × 300 mm) and UV-detection at 280 nm. As a mobile phase, a methanol-water linear-gradient under 20–100 % methanol (Sigma-Aldrich, Darmstadt, Germany) (5–35 min) was used at a flow rate of 1 mL/min. The final quantification was done based on an external calibration curve using standard IAA (Sigma-Aldrich, Darmstadt, Germany) **(Toumatia *et al.*, 2016)**. Results were expressed in mean (µg/mL) ± SD.

Tomato plant test with endophytic treatments for various durations under salt stress conditions

A tomato variety with fixed characters (*Solanum lycopersicum* L. 'Aicha') was chosen to study the potential of the endophytic bacteria to alleviate salt stress. The Aicha field cultivar, widely cultivated in Algeria, is characterized by a determinate growth. Tomato 'Aicha' seeds were cleaned twice in sterile distilled water. Surfaces were subsequently sterilized by immersion in 70% ethanol (Sigma-Aldrich, Darmstadt, Germany) for 1 min and then in 1% sodium hypochlorite for 10 min. Once disinfected, the seeds were washed five times with sterile distilled water and then aseptically air-dried in a sterilized absorbent paper **(Landa *et al.*, 2001)**.

The *in vitro* experiment was conducted using sterile Petrie plates, prepared by placing a sterilized absorbent paper disc in each plate. The plates were subsequently divided into 4 batches, each designed to test the effect of one NaCl concentration. Four different concentrations of NaCl solutions were prepared for irrigation: 50 mM (EC = 5.48 ± 0.03 mS/cm), 75 mM (EC = 7.93 ± 0.11 mS/cm), 100 mM (EC = 10.35 ± 0.21 mS/cm) and 125 mM (EC = 12.7 ± 0.14 mS/cm). The solutions were autoclaved.

The five selected bacterial strains were grown in TSB medium (150 mL) for 48h at 30 °C under vigorous shaking (120 rpm). The cultures were subjected to centrifugation at 6,000 rpm for 15 min. Cell pellets were resuspended in a separate sterile 0.9 % saline then shortly spun, followed by an adjustment of the bacterial charge to 1-2 × 10^7 CFU/mL. The bacterial suspensions were then distributed in 18 mL tubes, used to inoculate the disinfected tomato seeds **(Landa *et al.*, 2001)**. Bacterial seed treatment was carried out under aseptic conditions, by soaking 100 sterilized seeds in one of the adjusted bacterial suspension for each batch, for 3 different treatment durations: 1 h, 3 h, and 12 h, under shaking at 120 rpm, in darkness. The seeds of control batches were soaked in sterile saline for 1 h under agitation at 120 rpm **(Lee *et al.*, 2010)**.

Once treated seeds were ready, 20 seeds per plate were deposited in the previously prepared aseptic plates (4 replicates per batch); each seed spaced by at least 5 mm from the others. Once the seeds arranged in the Petri plates, batches were subjected to an initial watering of each plate with 5 mL of its corresponding saline solution. The plates remained closed during the first two days for optimal humidity; once the emergence of seedlings was initiated, the lids were removed. Each plate was watered every two days, from the day of the removal of the lid, by spraying the absorbent paper with 5 mL/plate, for a total period of 10 days **(Shahbaz *et al.*, 2011)**. The final germination percentages (%) and the fresh weights (g) of tomato seedlings were measured at the end of the experiment, expressed in mean ± SD.

Statistical analysis

The significance of differences in IAA production rates was investigated using Tukey's Honestly Significant Differences (HSD) multiple comparisons test at α= .05. The plant experiment had a completely randomized block design with four replications. Significance of the observed differences with controls (done on the raw measurements) was assessed using a two-tailed two-sample *t*-test (null hypothesis H_0: no significant difference between compared means; α= .05), after investigating the homoscedasticity (equality of variances) by the *F*-test. All statistical analyses were done using XLSTAT-Pro 7.5 software and built-in Excel functions. Charts were created using Plotly online software **(Plotly Technologies Inc. 2015)**, and arranged/composed under Adobe Illustrator CC 18.1.1 **(Adobe Inc., 2014)**.

RESULTS

Bacterial selection

This study led to a set of 25 endophytic strains, with different morphotypes and showing good growth at a salt concentration of 13 g/l (approx. 220 mM). This isolation has resulted in a bacterial group with close characters. However, the appearance does not constitute, alone, a rational basis for any objective selection. Thus, their ability to grow in a wide range of salinity (from 5 to 50 g/l) was investigated (Tab 1).

Table 1 Salt tolerance of the bacterial isolates

	NaCl concentrations (%)					
Isolates	0.5	1	2	2.5	3.5	5
BH1	+++	+++	+++	+++	+++	+
BH3	+	+	+	±	-	-
BH4	-	-	-	-	-	-
BH5	+	+	+	±	-	-
BH7	±	±	-	-	-	-
BH9	+++	+++	+++	+++	++	+
BH10	+	+	+	-	-	-
BH13	-	-	-	-	-	-
BH17	+++	-	-	-	-	-
BH18	+++	+++	+++	++	++	-
BH19	++	-	-	-	-	-
BH20	-	-	-	-	-	-
BH23	+	-	-	-	-	-
BH24	-	-	-	-	-	-
BH25	+++	+++	++	-	-	-
BH33	+++	+++	+++	+++	+++	+
BH34	+++	+++	+++	+++	+++	+
BH35	+++	+++	+++	+++	+++	+
BH39	+++	+++	+++	+++	+++	+++
BH40	+++	+++	+++	+++	+++	+++
BH46	+++	+++	+++	+++	+++	++
BH47	+++	+++	+++	+++	+++	-
BH48	+++	+++	+++	+++	+++	+
BH49	+++	+++	+++	+++	+++	++
BH50	+++	+++	+++	+++	+++	+

-: No growth, ±: very poor growth, +: poor growth, ++: moderate growth, +++: good growth

Assuming the use of PGPB inoculants for modulation of salt stress, it must have the ability to grow in saline soils, ensuring longer viability, in addition to other PGPB traits. According to such criteria, the top 5 representative endophytic isolates were selected, namely: BH1, BH39, BH40, BH49 and BH50.

Bacterial identification

The micromorphology of the five strains was described (after 24 h culture on TSA medium) in a fresh state and after Gram staining. The five isolates, BH1, BH39, BH40, BH49 and BH50 are Gram-positive, rod-shaped cells arranged in chains, mobile, with central endospores (ovoid/ slightly stick). The cells of the strains BH39, BH40, BH49, and BH50 are large, whilst those of BH1 are small. The five isolates were facultative anaerobic. The results of biochemical API20E galleries are reported in Table 2.

Table 2 API20E biochemical tests results of the selected bacterial strains.

API20E tests/ bacterial strains		BH1	BH39	BH40	BH49	BH50
Presence of	Galactosidase	-	-	-	-	-
	Arginine dihydrolase	-	-	-	-	+
	Lysine decarboxylase	-	-	-	-	-
	Ornithine decarboxylase	-	-	-	-	-
	Urease	-	-	-	-	-
	Gelatinase	+	-	-	-	+
	Tryptophan deaminase	+	+	-	-	+
Production of	H_2S	-	-	-	-	-
	Indole	-	-	-	-	-
	Acetoin	+	-	-	-	-
Use of	Citrate	-	-	-	-	-
	D-Glucose	-	-	-	-	-
	D-Mannitol	+	-	-	-	-
	myo-Inositol	-	-	-	-	-
	D-Sorbitol	-	-	-	-	-
	L-Rhamnose	-	-	-	-	-
	Sucrose	+	-	-	-	-
	D-Melibiose	-	-	-	-	-
	L-Arabinose	-	-	-	-	-
	Amygdalin	-	-	-	-	-

At the biochemical level, the strains appeared to converge (most of the tests were negative for the 5 isolates). Although strains BH39, BH40, BH49, and BH50 are physiologically very similar (except some variations in some tests), strain BH1 is distinct from these four strains by the production of acetoin and the use of D-mannitol and sucrose as a sole source of carbon. Strains BH39, BH40, BH49, and BH50, which are physiologically quite similar, seem be to phylogenetically (Figure 1, clade II) closely related as well (99.4 to 100 % of 16S rRNA gene similarity between them). These four strains showed similarity with species belonging to the *B. cereus* group, recently described by **Liu *et al.* (2017)**.

The phylogenetic analysis of the 16S rRNA genes of the 5 strains suggests their assignment to the *Bacillus cereus* group (Figure 1), which currently includes several species, that cannot be distinguished solely by their 16S rRNA genes **(Liu *et al.*, 2017)**. Thus, strains shall be named "*Bacillus* sp." until further taxonomic insights. Furthermore, the BH1 strain appears to be distinguishable from the remaining strains. This strain shows 98.7 % similarity with the closest species, *Bacillus paramycoides* KCTC 33709T (Figure 1, clade I). Given the current 16S rRNA gene similarity threshold (98.65 %) for species delineation (**Kim *et al.*, 2014)**, the strain *Bacillus* sp. BH1 is likely to be a novel species of the genus *Bacillus*.

The 16S rRNA gene sequences of the five strains have been deposited in the GenBank database and assigned the following accession numbers: KY910253 (*Bacillus* sp. BH1), KY910252 (*Bacillus* sp. BH39), MT704963 (*Bacillus* sp. BH40), KY910255 (*Bacillus* sp. BH49) and MT704962 (*Bacillus* sp. BH50).

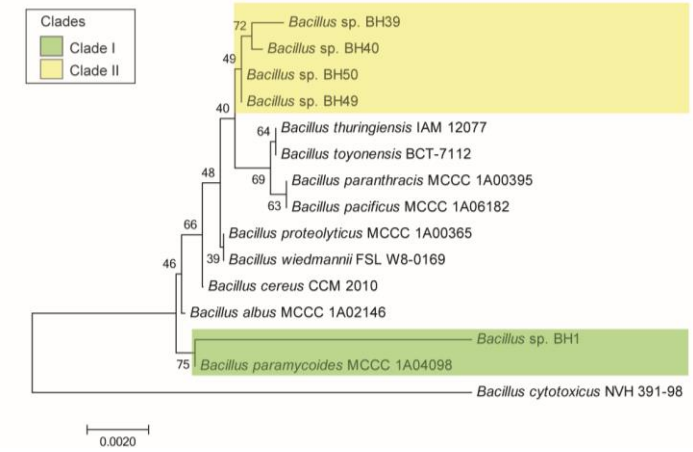

Figure 1 Minimum Evolution phylogenetic tree of endophytic strains *Bacillus* sp. BH1 (in clade I), *Bacillus* sp. BH39, *Bacillus* sp. BH40, *Bacillus* sp. BH49 and *Bacillus* sp. BH50 (in clade II) based on 16S rRNA gene sequences showing the relationship between the selected strains and the closest type-species of the *Bacillus* genus. The evolutionary history was inferred using the Minimum Evolution method **(Rzhetsky and Nei 1992)**. The optimal tree with the sum of branch length = 0.03444083 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches **(Felsenstein, 1985)**. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method **(Tamura *et al.*, 2004)** and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm **(Nei & Kumar, 2000)** at a search level of 1. The Neighbor-joining algorithm **(Saitou & Nei, 1987)** was used to generate the initial tree. The analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1463 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 **(Kumar *et al.*, 2016)**. The final tree was arranged using Adobe Illustrator CC 18.1.1 **(Adobe Inc. 2014)**.

IAA production

The quantification of IAA phytohormone (indole-3-acetic acid), known for its ability to promote the growth of plant roots, was undertaken by HPLC. Figure 2 shows the results, suggesting that strain *Bacillus* sp. BH40 has the highest rate of IAA phytohormone production with a production rate of 19.93 µg/mL. The lowest rate was observed for *Bacillus* sp. BH1 (14.62 µg/mL). No significant difference was spotted among the tested strains in terms of IAA production rates.

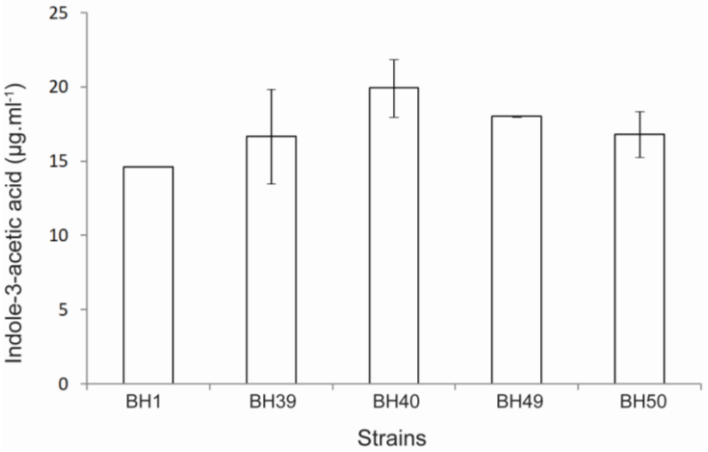

Figure 2 HPLC quantification of AIA (µg/mL) in batch cultures of the selected bacterial strains. Results expressed in mean ± SD. Means are not significantly different according to Tukey's Honestly Significant Differences (HSD) multiple comparisons test at $\alpha = 0.05$.

Plant test

For controls under salt stress, salinity appears to profoundly affect the morphology of tomato seedlings, with a shortening of the main root, a hardening of the collar, and slight browning of the latter in addition to a slight wilting of the upper part observed at the end of the experiment.

The effect of bacterial treatments on germination rates is shown in Figures 3, 4, and 5 for 1h, 3h, and 12 h respectively. In controls, germination rates are inversely proportional to salinity levels. For 1 hour treatment, significant enhancements of the germination rate were observed at different salinity levels.

From a strain perspective, *Bacillus* sp. BH40 and *Bacillus* sp. BH50 seem to be more efficient in improving germination when used for one hour.

Within a one hour treatment, *Bacillus* sp. BH40 lead to a significant enhancement of the germination rate at 50 mM (+13.75 %, p=0.04168), 100 mM (+13.75 %, p= 0.0320) and 125 mM (+21.25 %, p= 0.0018), while *Bacillus* sp. BH50 gave a significant improvement of germination rates at 50 mM (+17.5 %, p= 0.0271) and 100 mM (+27.5 %, p= 0.0371). For 3 hours of treatment, *Bacillus* sp. BH40 and *Bacillus* sp. BH50 gave no significant improvement, with a significant decrease (-40 %, p= 0.0010) in germination percentage at 50 mM for *Bacillus* sp. BH50.

A treatment of 12 hours using *Bacillus* sp. BH50 doesn't lead to any significant improvement (even worse at 50 mM and 75 mM, with a significant decrease in germination rates). At 12 hours, *Bacillus* sp. BH40 shows no significant improvement.

Meanwhile, *Bacillus* sp. BH39 and *Bacillus* sp. BH49 gave better results at relatively higher salinity levels (100 mM: +42.5 %, p= 0.0008 and +32.5 %, p= 0.0033, respectively) when used for 3 hours. *Bacillus* sp. BH39 exhibits a significant increase in germination rate at 125 mM for both 3 hours (+35 %, p= 0.023) and 12 hours (+32.5 %, p= 0.0049) treatment durations. The one-hour treatment using *Bacillus* sp. BH39 gave adverse effects on the germination rates, with a significant decrease at 75 mM, 100 mM, and 125 mM.

No significant effect on germination rates was observed using strain *Bacillus* sp. BH1, either positive or negative. Treatment duration effect on germination rates depends on the strain in question. For some strains, one hour is optimal (*Bacillus* sp. BH40, *Bacillus* sp. BH50), while 3 hours are best for other strains (*Bacillus* sp. BH39 and *Bacillus* sp. BH49).

For the same strain, different durations can either alleviate salt stress in a significant way or cause an adverse effect, decreasing significantly the germination rate as observed with the tested endophytes.

We insist on suggesting that the optimal duration of bacterial treatments should be determined, as we proved that it might significantly vary according to the bacterial strains in question, and to the salinity levels for which it is intended.

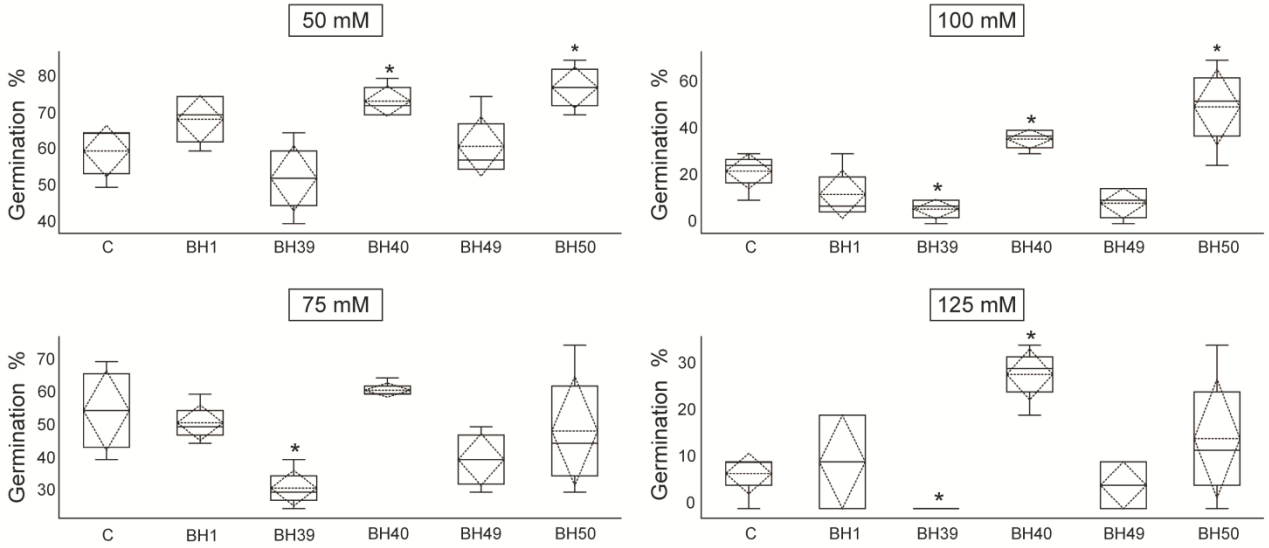

Figure 3 Germination rates (absolute value = 100 %) of tomato 'Aicha' seeds at 50 mM, 75 mM, 100 mM, and 125 mM (NaCl) after treatment with endophytic strains *Bacillus* sp. BH1, *Bacillus* sp. BH39, *Bacillus* sp. BH40, *Bacillus* sp. BH49 and *Bacillus* sp. BH50 for 1 h. For each box: upper square line = upper quartile (q3), bottom square line = lower quartile (q1), middle square line =median, upper whisker = highest observed value, bottom whisker = lowest observed value; dashed middle line = mean, dashed diamond= standard deviation. *: Significant difference against control according to a two-tailed two-sample *t*-test at α = .05.

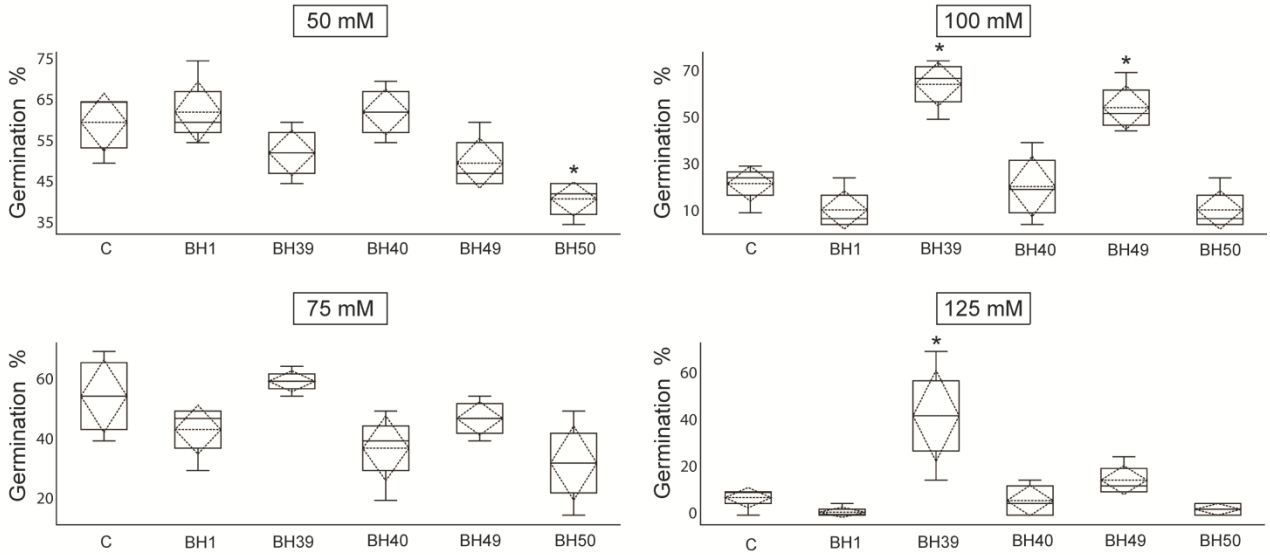

Figure 4 Germination rates (absolute value = 100 %) of tomato 'Aicha' seeds at 50 mM, 75 mM, 100 mM, and 125 mM (NaCl) after treatment with endophytic strains *Bacillus* sp. BH1, *Bacillus* sp. BH39, *Bacillus* sp. BH40, *Bacillus* sp. BH49 and *Bacillus* sp. BH50 for 3h. For each box: upper square line = upper quartile (q3), bottom square line = lower quartile (q1), middle square line =median, upper whisker = highest observed value, bottom whisker = lowest observed value; dashed middle line = mean, dashed diamond= standard deviation. *: Significant difference against control according to a two-tailed two-sample *t*-test at α = .05.

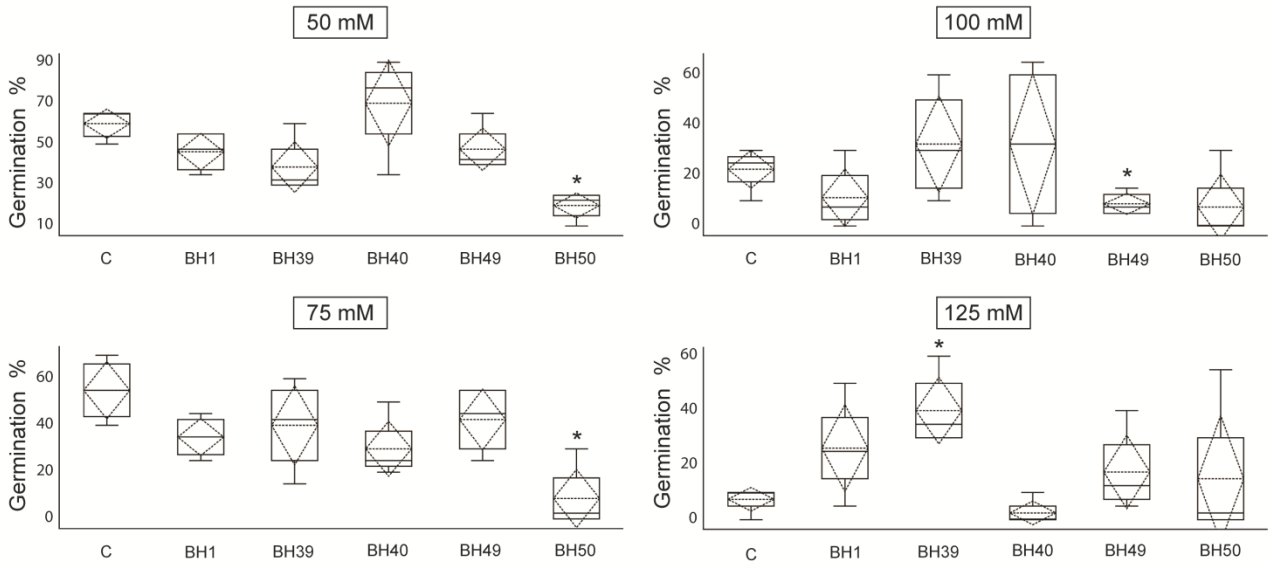

Figure 5 Germination rates (absolute value = 100 %) of tomato 'Aicha' seeds at 50 mM, 75 mM, 100 mM, and 125 mM (NaCl) after treatment with endophytic strains *Bacillus* sp. BH1, *Bacillus* sp. BH39, *Bacillus* sp. BH40, *Bacillus* sp. BH49 and *Bacillus* sp. BH50 for 12h. For each box: upper square line = upper quartile (q3), bottom square line = lower quartile (q1), middle square line =median, upper whisker = highest observed value, bottom whisker = lowest observed value; dashed middle line = mean, dashed diamond= standard deviation. *: Significant difference against control according to a two-tailed two-sample *t*-test at α = .05.

The following part concerns the plants treated with *Bacillus* sp. BH40 and *Bacillus* sp. BH50. Plants treated with the remaining strains did not survive. Figures 6, 7, and 8 shows the average fresh weights of the batches treated with *Bacillus* sp. BH40 and *Bacillus* sp. BH50 for 1h, 3h, and 12 h respectively.

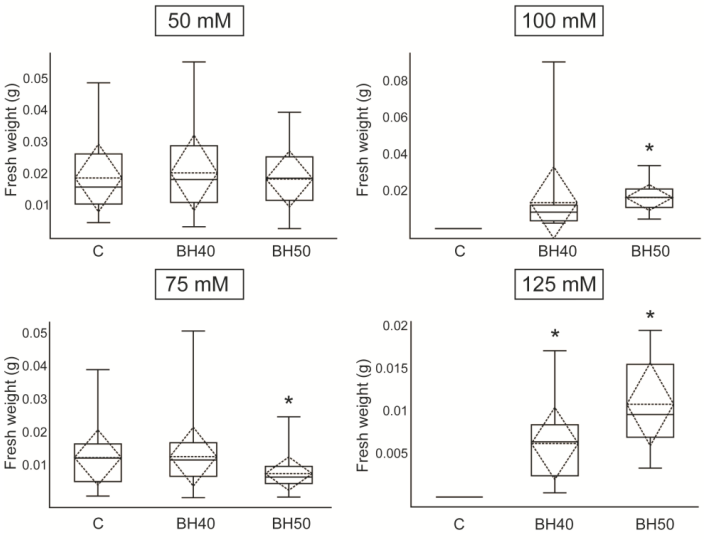

Figure 6 Fresh weights of tomato plants at 50 mM, 75 mM, 100 mM, and 125 mM after treatment with endophytic strains *Bacillus* sp. BH40 and *Bacillus* sp. BH50 for 1 h. For each box: upper square line = upper quartile (q3), lower square line = lower quartile (q1), middle square line = median, upper whisker = highest observed value, lower whisker = lowest observed value; dashed middle line = mean, dashed diamond = standard deviation. *: Significant difference against control according to a two-tailed two-sample *t*-test at α = .05.

At 50 mM, the best result was obtained with *Bacillus* sp. BH50 after a 3 hours bacterization, leading to a significant improvement of fresh weight of 31.88 % (p= 0.0128). At 75 mM, the best improvement was observed in plants treated for 3 hours with *Bacillus* sp. BH50 as well, with a significant amelioration of 90.38 % (p= 2.4534E-06). At 100 mM and 125 mM, control plants died due to the accumulation of high levels of salt, while those treated with *Bacillus* sp. BH40 and *Bacillus* sp. BH50 survived. Thus, improvement percentages won't be mentioned at these concentrations.

Top fresh weight values at 100 mM and 125 mM were recorded with *Bacillus* sp. BH50, after a one-hour treatment. A deleterious effect on fresh weight was observed for *Bacillus* sp. BH50 at 75 mM (one hour treatment: -38.42 %, p= 0.0020) and 50 mM (12 hours treatment: -64.93 %, p= 1.06963E-08), while this negative effect was not observed for *Bacillus* sp. BH40 under any circumstance. *Bacillus* sp. BH40 achieved a significant positive effect on fresh weight values at 125 mM (one hour, p= 0.0036) and 100 mM (3 hours: p= 0.0025, and 12 hours: p= 7.7934E-05) compared to dead plants in control.

Overall, the highest fresh weight improvements were observed in plants treated for 3 hours. Just like chemicals, dose and exposition to biofertilizers might affect the final results, balancing from intended improvement to unwanted deleterious effect. Moreover, microbial solutions for salt stress mitigation should be specific to a certain level of salinity, as our data showed that some strains might be effective at a salt concentration while ineffective at another concentration.

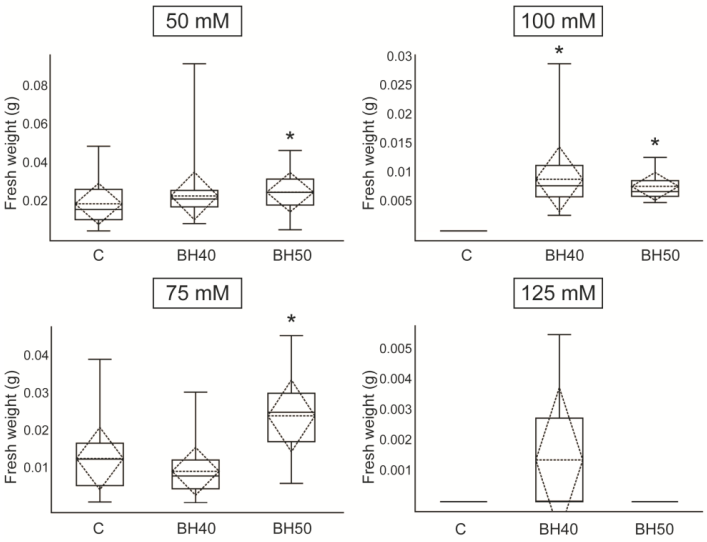

Figure 7 Fresh weights of tomato plants at 50 mM, 75 mM, 100 mM, and 125 mM after treatment with endophytic strains *Bacillus* sp. BH40 and *Bacillus* sp. BH50 for 3h. For each box: upper square line = upper quartile (q3), lower square line = lower quartile (q1), middle square line = median, upper whisker = highest observed value, lower whisker = lowest observed value; dashed middle line = mean, dashed diamond = standard deviation. *: Significant difference against control according to a two-tailed two-sample *t*-test at α = .05.

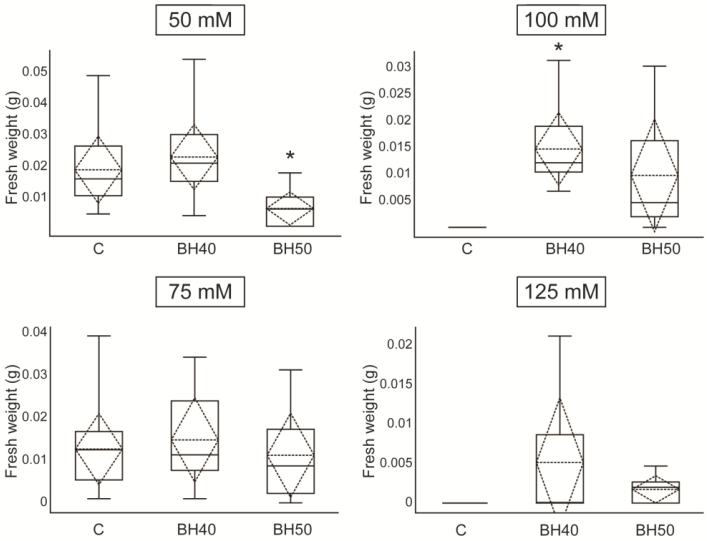

Figure 8 Fresh weights of tomato plants at 50 mM, 75 mM, 100 mM, and 125 mM after treatment with endophytic strains *Bacillus* sp. BH40 and *Bacillus* sp. BH50 for 12h. For each box: upper square line = upper quartile (q3), lower square line = lower quartile (q1), middle square line = median, upper whisker = highest observed value, lower whisker = lowest observed value; dashed middle line = mean, dashed diamond = standard deviation. *: Significant difference against control according to a two-tailed two-sample *t*-test at α = .05.

DISCUSSION

Overall, the highest fresh weight improvements were observed in plants treated for 3 hours. Just like chemicals, the dose and exposure to biofertilizers can significantly impact the final results **(Johnson *et al.*, 2018)**. It is important to carefully consider the application duration and concentration to achieve the desired improvements while minimizing any potential deleterious effects **(Garcia *et al.*, 2019)**. Thus, understanding the optimal treatment duration and dosage is crucial in ensuring the effectiveness of biofertilizers in promoting plant growth and avoiding any negative consequences. Further studies have demonstrated that excessive exposure or improper application of biofertilizers can lead to adverse effects on plant development and physiology **(Nguyen *et al.*, 2016).** Therefore, it is essential to strike a balance between the intended improvement and the potential unwanted consequences when utilizing biofertilizers for plant growth enhancement.

Moreover, microbial solutions for salt stress mitigation should be tailored to specific levels of salinity, as our data showed that certain strains may be effective at one salt concentration while proving ineffective at another. The effectiveness of microbial solutions in mitigating salt stress is highly dependent on the salinity tolerance and adaptability of the selected strains **(Kang *et al.*, 2019)**. Different salinity levels can impose varying degrees of stress on plants, and the response of microbial strains to salt stress may vary accordingly **(Ahmad *et al.*, 2020)**. Therefore, it is crucial to carefully select and test microbial strains that are specifically adapted to the targeted salinity level to achieve optimal results in salt stress mitigation. Recent studies have highlighted the importance of matching the salinity tolerance of microbial strains with the salt concentration in the target environment for successful stress alleviation in plants **(Sharma *et al.*, 2021)**. This approach ensures that the selected strains can effectively colonize the rhizosphere and establish beneficial interactions with the host plants, ultimately enhancing their salt tolerance and growth under the specific salinity conditions.

Wild plants rhizosphere is undoubtedly perceived as one of the best sources for the isolation of PGPB. This is due to the strong selective pressure exerted by the plants by selecting, among others, the beneficial bacteria. During a PGPB screening, soil type, plant species, seasons, and plant physiological moments are parameters that must be taken into consideration to ensure the isolation of targeted rhizobacteria, supposedly beneficial **(Lugtenberg, 2015)**. It is generally accepted that the rhizosphere is the volume of soil surrounding the roots (1-3 mm around the root and the soil adhering to the root). Depending on the type of study, the root containing endophytic bacteria is included in the late definition. PGPB isolation strategies generally go through a step called "extraction" which, as its name suggests, meant to extract the desired agent from the rhizosphere. In this present study, the extraction was performed by a single physical treatment (grinding roots), followed by vigorous homogenization. Chemical extraction methods may be combined with physical methods, and these can be divided into three categories: shaking, mixing (homogenizing or grinding), and ultrasonics **(Ahmad *et al.*, 2008)**.

The isolation of a pure culture of a target microorganism is often the first step in an applied microbiology project. Selective media and growth conditions developed for the isolation and culture of specific groups of bacteria and archaea are extensively described in the literature. In our study, the phenotypic tests consistently identified the Bacillaceae family, a group widely recognized for its proven traits as plant growth-promoting bacteria (PGPB) **(Tiwari *et al.*, 2019)**. Furthermore, the analysis of 16S rRNA gene sequences indicated that all isolates belonged to the *Bacillus cereus* group, a subdivision of the *Bacillus* genus that currently comprises 12 closely related species **(Liu *et al.*, 2017)**. This group includes well-known species such as *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus weihenstephanensis*, *Bacillus pseudomycoides*, and recently identified species such as *Bacillus gaemokensis*, *Bacillus manliponensis*, *Bacillus cytotoxicus*, *Bacillus toyonensis*, *Bacillus bingmayongensis*, and *Bacillus wiedmannii*. Additionally, genomic evidence has expanded this group to include nine additional species, namely, *Bacillus paranthracis*, *Bacillus pacificus*, *Bacillus tropicus*, *Bacillus albus*, *Bacillus mobilis*, *Bacillus luti*, *Bacillus proteolyticus*, *Bacillus nitratireducens*, and *Bacillus paramycoides* **(Liu *et al.*, 2017)**. Based on our findings, strains BH40, BH49, and BH50 exhibited close genetic relatedness to *Bacillus thuringiensis*, *Bacillus toyonensis*, *Bacillus paranthracis*, and *Bacillus pacificus*, while strain BH1 appeared to be closely related to *Bacillus paramycoides*. The members of the *Bacillus cereus* group display a wide range of adaptability to different lifestyles, as evidenced by their diverse interactions with humans. Some strains and toxins are highly regarded for their potential applications as probiotics, biological plant protection agents, and insecticides, offering numerous benefits in agriculture and environmental management **(Ceuppens *et al.*, 2013)**. Conversely, other strains and toxins are closely monitored and controlled due to their ability to cause food poisoning, spoilage of food products, infections, and even anthrax disease **(Ehling-Schulz *et al.*, 2015)**.

We undertook the exploration of IAA production by the selected strains during this study. The production of secondary metabolites is recognized as an important aspect of promoting plant growth. Phytohormones play a critical role in regulating plant growth and development. As such, and after a selection of the most interesting isolates, a characterization of PGPB potential was carried out through the production of a plant hormone (auxin), namely indole-3-acetic acid (IAA). This plant hormone is associated with PGPB capacity to stimulate root growth in host plants, once inoculated with IAA producers resulting in a greater root surface and accessibility for more nutrients **(Barazani & Friedman, 1999; Patten & Glick, 2002)**. Moreover, IAA application to salinity stressed tomato plants has been proven to rescue the plants and induce significantly positive effects on growth and yield of tomato plants **(Alam *et al.*, 2019)**.

In the present study, tomato (*Solanum lycopersicum* L.) was chosen as a model plant for its economical importance, and its sensitivity to salinity. Its growth is greatly affected by environmental stresses such as low temperatures, drought, or high salinity. From an agricultural point of view, these types of stress, in addition to biotic pests, are the most limiting factors **(Cuartero *et al.*, 2006)**. This work focused on investigating the root endophytic bacteria associated with selected steppe plants, specifically *Atriplex halimus* L. and *Tamarix aphylla* L. Root colonization is a crucial process through which bacteria, introduced via seeds, vegetative propagation, or present in the soil, establish and propagate within the roots. This colonization process encompasses various parts of the rhizosphere, including the interior of the root, the root surface (rhizosphere), and the surrounding soil **(Landa *et al.*, 2001)**. The selected bacterial isolates were obtained from the inner tissues of the plants, operating under the assumption that bacteria residing inside the plants, without inducing detrimental effects, may potentially confer beneficial impacts. The exploration of endophytic bacteria residing within plant tissues holds promise for uncovering novel associations and understanding their ecological roles **(Hardoim *et al.*, 2015)**. By residing within the plants, these endophytes can influence plant growth, nutrient uptake, and stress tolerance **(Hallmann *et al.*, 1997)**.

The comprehensive analysis of the in vitro tests conducted in this study highlights the remarkable potential of *Bacillus* sp. BH40 and *Bacillus* sp. BH50 as highly promising strains. These strains exhibited significant positive effects on multiple parameters, including germination percentage enhancement (up to +27.5%) and fresh weight improvement (up to +90.38%). Such outcomes underscore the efficacy of these strains in promoting plant growth and development under stress conditions. Moreover, the application of *Bacillus* sp. BH40 and *Bacillus* sp. BH50 treatments demonstrated their ability to confer protection to tomato 'Aicha' seedlings against high salinity levels, a challenge that led to the demise of control plants. This protective effect suggests that these strains possess mechanisms to mitigate the detrimental impacts of salinity stress on plant growth. The findings from this study align with previous research highlighting the plant growth-promoting potential of *Bacillus* strains. Several studies have reported the beneficial effects of *Bacillus* spp. on seed germination, plant growth, and stress tolerance in various crops **(Berg et al., 2017; Ma et al., 2016; Khan et al., 2022)**. The ability of these strains to enhance plant performance under stressful conditions further emphasizes their potential as biofertilizers for sustainable agriculture.

Soaking seeds in different solutions at a different concentration or pure water for the different duration to enhance imbibition capacity and pre-germination metabolism and to subsequently induce tolerance in them to stressed conditions with potential improvement in growth, vigor, and yield is commonly known as ‘seed priming’ **(Varier *et al.*, 2010; Sano *et al.*, 2017).** Biopriming with plant growth promoting bacteria can mitigate the adverse effects of abiotic stresses including salinity on crops **(Majeed *et al.*, 2018; Majeed *et al.*, 2019; Fiodor et**

al., 2023). **Bruce *et al.* (2007)** reported that phytohormones and regulatory substances (auxins, gibberellins, abscisic acid, ethylene, jasmonic acids, etc.) are possibly correlated with responses of plants to various stresses. Seed biopriming proved to trigger the role of these substances which enable plants to perform in a better way under the stressful environment. Seed biopriming has been found to trigger the action of these substances, enabling plants to perform better under stressful environmental conditions. The mechanisms behind seed biopriming involve the stimulation of morphogenesis and plant immunity through various processes, including the production of phytohormones, induced expression of plant growth-promoting genes, increased nutrient uptake, mycoparasitism, antibiosis, induced phenolic production, activation of antioxidant systems, and systemic defense activation **(Singh *et al.*, 2018; Hoque *et al.*, 2023)**. These findings align with previous research demonstrating the positive effects of seed biopriming on plant growth and stress tolerance. Studies have highlighted the role of biopriming in enhancing crop performance under adverse conditions, including salinity stress **(Fiodor *et al.*, 2023)**. The understanding of these mechanisms and their application in seed biopriming provides valuable insights for sustainable agriculture and the development of strategies to improve crop productivity in challenging environments.

It is noteworthy to highlight that the beneficial effects of bacterial treatment depend on a variety of factors, including the effectiveness of the strain, the cell charge, the quantity of the microbial formulation, the duration of the treatment, the species of the plant, the soil composition, etc. **(Tsavkelova *et al.*, 2006)**. The phenomenon of seed priming involves the induction of reversible and short-duration effects that aim to create a memory in seeds, preparing them for potential stressful conditions in the future **(Lämke & Bäurle, 2017)**. Studies on different plants showed that optimal soaking times depend on the plant species in question. The duration of seed priming, along with the optimal concentration of the priming agent, plays a crucial role in determining the success of germination and seedling establishment **(Ibrahim, 2019)**. Several studies have discussed the impact of priming duration for various priming agents. For instance, **Hayat et al. (2022)** investigated the effect of plant-derived smoke exposure on wheat and found significant results with shorter exposure times of 1 hour and 2 hours compared to prolonged exposure times of 3 hours and 4 hours. This suggests that the duration of priming can influence the outcomes of germination. Similarly, the influence of soaking duration on germination and seedling growth of *Lycopersicum esculentum* was evaluated by **Sabongari & Aliero (2004)**. They observed that treatment duration of 36 hours did not lead to a significant improvement in germination, whereas the maximum germination rate was achieved with treatment duration of 24 hours. Moreover, **Sabongari & Aliero (2004)** reported that longer soaking durations for tomato seeds were ineffective in promoting seed germination. Moreover, **Singh *et al.* (2014)** found that priming onion seeds for 24 h significantly enhanced seed germination (81.6 %) as compared to 12 h, 36 h, or 48 h duration, and increasing priming duration from 2 days to 6 days led to a significant decrease in seed germination. Moreover, priming for 36 h gave the fastest germination time for maize seeds among 12 h to 48 h priming duration **(Hacisalihoglu *et al.*, 2018)**. These findings highlight the importance of optimizing the duration of seed priming treatments to achieve desired germination outcomes. Understanding the relationship between priming duration and its effects on germination rates can contribute to the development of effective seed priming strategies for improving crop performance under various stress conditions. Generally, the success of seed priming is influenced by the duration of priming and vegetable species **(Ibrahim, 2019)**.

Through this study, the demonstrated effectiveness of the selected strains *Bacillus* sp. BH40 and *Bacillus* sp. BH50 suggests these endophytes as a promising prospect for the application of such microbial auxiliaries in salt stress alleviation, by improving resistance profiles/salinity tolerance of important crops. Moreover, it opens perspectives for other effective PGPB endophytes from harsh environments, considering the important ecological potential of the extremely diversified Algerian territory, especially its vast arid regions **(Sabaou *et al.*, 1998)**.

CONCLUSION

As a conclusion, tomato 'Aicha' is favorably disposed to respond positively to the tested endophytic bacteria. Treatment durations affect final result depending on the tested endophyte, and the salinity level, which leads to the necessity of duration optimization according to the mentioned factors. *Bacillus* sp. BH40 and *Bacillus* sp. BH50 are the most effective in protecting and promoting the growth of tomato under high salinity conditions, with a significant advantage for *Bacillus* sp. BH50 in terms of fresh weight improvement, with an optimal treatment time of 3 hours at 50 mM, 75 mM and 100 mM, and 1 hour at 125 mM.

Acknowledgments: We would particularly like to dedicate this work to the memory of our Professor Nasserdine SABAOU, who we lost in 2019. We shall proudly remember him for his contribution to the taxonomy of bacteria from arid regions, especially Actinobacteria, for which he dedicated his entire life.

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