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Seed Inoculation with Halotolerant Strains Enhance Brassicaceae Seedling Establishment Under Saline Conditions

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Abstract: Soil salinity inhibits germination and seedling establishment, causing patchy crop stands, uneven growth, and poor yields. This study aims to evaluate the early-stage salinity tolerance of Brassicaceae seeds inoculated with plant growth-promoting bacterial (PGPB) strains (E1 and T7) isolated from saline soils. Non-inoculated and inoculated seeds of *Lobularia maritima*, *Sinapis alba*, and *Brassica napus* were cultivated under control and salinity conditions, first in agar plates to assess a germination inhibitory concentration of salt for each species and later in soil irrigated with water containing 0 or 75 mM NaCl. Our results indicate that T7 was the only strain able to increase the germination of *L. maritima* under saline conditions. However, an increase in shoot biomass, root length, and number of branches was observed in *L. maritima* and *S. alba* plants inoculated with T7 and in *B. napus* with E1. Concomitantly, those seedlings exhibited less oxidative damage and greater capacity to balance plant reactive oxygen species production. This study suggests that inoculation of seeds with halotolerant PGPB strains is a suitable strategy for improving the negative effects of salinity in the early stages. Nonetheless, the observed specific plant–host interaction highlights the need for establishing tailored PGPB–crop associations for specific unfavourable environmental conditions.

Keywords: salinity; PGPB; Brassicaceae; seed inoculation; seedling establishment

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1. Introduction

Salinity stands out as one of the most critical environmental stresses limiting yields, because most crop species are highly salt-sensitive [1]. Seedling emergence is a critical step that determines whether crop yield is significantly compromised by high soil salinity [2]. Such conditions decrease the osmotic potential around the seeds, reducing water uptake and consequently delaying or inhibiting germination [3]. This interference is linked to a decrease in gibberellins levels (GA) and the induction of abscisic acid (ABA) production [4–6]. Simultaneously, the toxic accumulation of Na^+ and Cl^- inhibits K^+ and Ca^{2+} uptake, disrupting ion balance and pH homeostasis [7]. The resulting osmotic and ionic stresses cause the overaccumulation of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO·) [8]. Although ROS are formed as natural by-products of the metabolism of plants and have a positive impact on plant growth, promoting cell elongation in the hypocotyl area during the initial stage of radicle emergence [9], the uncontrolled ROS overaccumulation under salinity conditions can be extremely harmful to germinating seeds [10]. ROS can damage nucleic acids, impacting the DNA of the embryo, and compromise membrane integrity, leading to the production of malondialdehyde (MDA), a final product of membrane lipid peroxidation that is commonly used as an indicator of oxidative stress in plants [8,11,12]. Moreover, ROS-induced programmed cell death (PCD) can be a further consequence of salinity stress in sensitive plants [13].

The establishment of a mutualistic relationship between plants and their microbiomes can be an effective strategy to help seeds to cope with salinity stress [14,15]. Among the plethora of microorganisms that inhabit the plant rhizosphere, a group named plant growth-promoting bacteria (PGPB) are gaining increasing attention for their ability to enhance plant adaptation to adverse soil conditions [16]. This interest has been intensified due to global climate change and its associated rise in abiotic stress severity, threatening agricultural productivity [17]. In this context, PGPB are key components of the soil ecosystem that improve plant health and diversity maintenance [18–20]. Some examples of the most widely studied PGPB genera are, among others, *Acinetobacter* spp., *Azospirillum* spp., *Azotobacter* spp., *Bacillus* spp., *Pseudomonas* spp. and *Rhizobia* spp. [21]. Besides the well-known PGP mechanisms that improves nutrient availability (solubilizing unavailable sources of phosphate, synthetizing specialized high-affinity iron chelator as siderophores to enhance Fe availability and fixation of atmospheric nitrogen) [22,23], different physiological responses can be observed when PGPB interacts with plants growing under unfavourable growth conditions [24]. In this regard, they can modulate the synthesis of plant hormones, phytochemicals, and secondary metabolites; increase the antioxidant response; and induce osmolytes production and the modulation of stress gene expressions [25–27]. These beneficial microorganisms usually present more than one PGP mechanism, constituting an ecological and economical alternative to the use of chemical fertilizers and pesticides [28]. PGPB like certain *Pseudomonas* spp. can promote seed germination by synthesizing phytohormones such as indole-3-acetic acid (IAA) and/or by reducing ABA accumulation [19,25,29]. The excessive accumulation of stress-induced ethylene under salinity can be mitigated with PGPB inoculation, as certain strains produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme responsible for the degradation of ACC, the precursor of ethylene [30]. Specifically, in plants growing under salinity stress, the application of beneficial bacteria can provide defense against oxidative stress by improving nutrient availability, reducing MDA concentrations, increasing the activity of antioxidant enzymes, and promoting proline biosynthesis (a well-known osmoprotectant) [20,31,32]. However, different environmental stresses, such as salinity, can alter the composition of root exudates, affecting the interaction with PGPB, or directly impact PGP mechanisms, leading to a reduction in plant yield [31,33–35]. Therefore, the choice of the best "micro" partner to plants is decisive in obtaining optimal plant performance. Thus, an important challenge is to select microorganisms that are environmentally friendly, show high competitiveness in the soil without affecting other beneficial rhizosphere microorganisms, and tolerate the environmental conditions [36].

The Brassicaceae family comprises valuable crops with high economic and agronomic significance, providing important human and animal food resources, including fodder, oilseed, vegetables, and condiments [37]. In addition, brassicas are used to produce fuels and lubricants, in bioremediation programs, or even as ornamental plants. Although the members of this family do not engage in symbiotic interactions with rhizobia, it has been shown that its interaction with different epiphytic or endophytic PGPB has positive results, as observed in *Brassica napus*, *Camelina sativa*, and *Arabidopsis thaliana* [38–41]. However, studies regarding the interaction between PGPBs and Brassicaceae members are scarce. To improve the resilience of this globally relevant crop family, further studies about abiotic stress responses and mitigation strategies are required. Thus, the aim of this research was to evaluate the effects of the inoculation of valuable brassica seeds with two salt-tolerant PGPB strains on the germination rate, seedling establishment, and oxidative stress responses when exposed to high-salinity conditions. For this purpose, we selected canola (*Brassica napus*) and yellow mustard (*Sinapis alba*) due to their importance as sources of vegetable oil and their low tolerance to drought, salinity, and high temperatures [42]. As a contrasting salt-tolerant Brassicaceae species, sweet alyssum (*Lobularia maritima*) was included in the study. Although this ornamental halophyte can tolerate high levels of salt, under conditions where salinity impairs seed germination, it becomes as salt-sensitive as glycophytes during the early stages of its life cycle [43].

2. Material and Methods

2.1. Plant Material and Growth Conditions

In this study, seeds from commercial lines of *L. maritima* (Vilmorin, Paris, France), *B. napus* (Grup Morera, Les Preses, Girona, Spain), and *S. alba* (Semillas Batlle, Molins de Rei, Barcelona, Spain) were used. Prior to sowing, surface sterilization was performed by soaking seeds in commercial bleach (10% (*v*/*v*) for *B. napus* and *S. alba* or 30% (*v*/*v*) for *L. maritima*) and a drop of Tween-20 for 15 min. After several washings with autoclaved 18 MΩ Milli-Q water, seeds were left for 2 days at 4 °C to stratify and then used for the following experiments. For in vitro and soil gemination assays, plants were grown in a controlled chamber with a 150 mmol/m^2s of light intensity, a 16 h light/8 h dark photoperiod, a 25 °C day/night temperature, and 40% humidity.

2.2. Bacterial Strains

The native *Pantoea* sp. (T7) and *Pseudomonas* sp. (E1), isolated from the Mediterranean coastal soils of Tossa de Mar (41.71499, 2.93111; soil [Na^+] = 91.35 ± 15.73 mg/g) and L'Escala (42.12571, 3.12775; soil [Na^+] = 112.34 ± 23.48 mg/g), were selected for seed inoculation assays. Both bacterial strains were classified as PGPB, as they showed different PGP mechanisms related to nutrient uptake (P, Fe, N) [44]. The bacteria were grown in Luria Bertani (LB) medium on an orbital shaker at 150 rpm at 25 °C. Growth was measured turbidimetrically at an absorbance of 620 nm, and the number of viable cells was determined by colony-forming units (cfu) using the drop-plate method [45]. The long-term preservation of the microorganisms was carried out by taking aliquots of cultures in the late logarithmic phase supplemented with sterile glycerol until they reached a final concentration of 40%. The prepared suspensions were stored at −20 °C.

2.3. Salt Screening Germination Assay

Twenty seeds of each species were sown in square plates containing ½ MS medium (0.6% agar, pH 6) supplemented with 0, 50, 75, 100, 150 mM, or 200 mM of NaCl. Five days after sowing, the germination rate (considered as the number of seeds with visible radicle protrusion) was measured. Three independent replicates were performed. The experimental NaCl concentration selected to test the effects of PGPB inoculation on seed germination was the highest concentration that caused more than a 20% but less than a 50% reduction in the germination rate.

2.4. In Vitro Germination Assay

Surface-sterilized seeds were coated with 0.6% gum Arabic and rolled into each bacterial suspension (10^9 cfu/mL, DO = 2) for 20 min [46]. Each bacterial suspension was grown for 16 h (until the end of the exponential phase) in a 125 mL Erlenmeyer flask with LB culture medium. Then, they were centrifuged at 10,000× *g* for 15 min, resuspended in an equal volume of physiological solution, and inoculated into each seed species. For non-inoculated seeds, gum Arabic was supplemented with autoclaved deionized water ($_dH_2O$). Prior to the germination assay, the adhesion capacity to the seeds of both PGPB were measured following the methodology described in Smith and Wollum [47], with slight modifications for seeds. Twenty seeds of each species were sown in ½ MS plates supplemented with 75 mM NaCl for *B. napus* or 150 mM NaCl for *L. maritima* and *S. alba* (salt plates). Plates without salt addition were maintained as controls. Germination rates were measured 5 days after sowing. Three independent replicates were performed.

2.5. Soil Germination Assay

Fifty seeds of each species, either non-inoculated (N/I) or inoculated with E1 or T7, were sown in trays containing a sterilized mixture of peat, sand, and perlite (1:1:1 volume). Trays were irrigated with $_dH_2O$ devoid of salt (control) or supplemented with 75 mM NaCl from day 0. Germinated seeds and the number of established seedlings (plants with true leaves) were counted 5 and 10 days after sowing. Shoot length was measured every 5 days.

Fifteen days after sowing, plants were harvested, and root length and fresh weight were measured. The roots of five plants per treatment and inoculation were scanned (Epson Expression 10,000 XL), and root morphology was analyzed using the WinRHIZO v. 2009c software (RRID:SCR_017120) (Figure S1). Shoots were oven-dried at 60 °C for two days, and the dried weight (DW) was determined. Roots were pooled in groups of five, frozen in liquid nitrogen, and stored at −80 °C until further use. The salinity tolerance index (STI) was calculated following Saade et al.'s formula [48].

$$STI = \frac{DWc}{DWav} \times \frac{DWs}{DWav}$$

2.6. Leaf Na^+ and K^+ Determination

Dried samples (0.05 g) were pre-digested overnight in Pyrex tubes using 15 mL HNO_3 69%, followed by an open-air digestion at 110 °C for 2 h in a hot-block digestion system (SCI54-54-Well Hot Block, Environmental Express, Charleston, SC, USA). Concentrations of Na^+ and K^+ were determined by a flame photometer (Jenway, PFP7, Chicago, IL, USA).

2.7. ROS Production

Hydrogen peroxide levels and lipid peroxidation were determined in the roots of brassicas following Alexieva et al. [49] and Heath and Packer [50], with modifications. Pooled roots (0.1 g) were ground in 1 mL of 0.1% trichloroacetic acid (TCA) (Sigma, Darmstadt, Germany) and centrifugated at 10,600× *g* for 10 min, and then the supernatant was used for each determination. For the H_2O_2 determination, a 160 µL aliquot of the above-described extract was mixed with 160 µL of 100 mM K-phosphate buffer, pH 6.8, and 680 µL 1 M KI (Sigma). The reaction mixture was incubated for 1 h in darkness. The quantification was measured using a plate spectrophotometer (TECAN) at 390 nm. The blank probe consisted of 0.1% TCA. Three technical replicates per sample were measured. The amount of hydrogen peroxide was determined using a standard curve that contained known concentrations of H_2O_2.

Oxidative damage was determined by the production of Thiobarbituric Acid-Reactive Substances (TBARs) by mixing 500 µL of 0.5% TBA (Sigma) in 20% TCA with 500 µL of an aliquot root extract. The solution was heated at 95 °C for 25 min, cooled down in ice for 10 min, and centrifuged for 5 min at 3800× *g* to remove turbidity. Reaction mixture absorbance was measured using a plate spectrophotometer (TECAN) at 532 nm, and non-specific absorption was measured at 600 nm. The blank probe consisted of 0.1% TCA. Three technical replicates per sample were measured. The TBARs concentration was determined using a calibration curve containing known concentrations of 1,1,3,3-Tetraethoxypropane.

2.8. Root Viability Assay

Viability of the root tips was determined using fluorescein diacetate–propidium iodide (FDA–PI). Root tips of fresh harvested 15-day-old seedlings (from soil germination assay) were stained for 3 min in 1 mL of FDA (12.5 µg/mL), washed twice in DPBS buffer (140 mM NaCl, 6 mM Na_2HPO_4, 4 mM KH_2PO_4, pH 7.4), and then stained for 10 min in PI (5 µg/mL). Extra dye was removed by washing in DPBS buffer. Root fluorescence was observed using epifluorescence microscopy (excitation filter: 450–490 nm; emission filter: 520 nm).

2.9. Data Analysis

The data were analyzed using RStudio (base R.4.3.3). Graphs were designed using the ggplot2 package [51]. Differences among salt treatments were analyzed using *t*-tests, while differences among bacterial inoculation were analyzed using one-way ANOVA, considering $p < 0.05$ as significantly different according to the Duncan test. One-way repeated-measures MANOVA was used to test for differences in shoot length among bacterial inoculations over time. All statistical analyses are available at Dataset S1.

3. Results and Discussion

3.1. Salt Screening Germination Assay and Effects of PGPB on Germination

Salinity tolerance levels among Brassicaceae species differ widely depending on their genetic background and evolution. Seed germination decreased as salinity increased in the three Brassicaceae species tested, but they showed differences in their sensibility to salt stress (Figure S2. *Lobularia maritima* was the most salt-tolerant species, followed by *S. alba*, while *B. napus* was the most sensitive (Figure 1). *Lobularia maritima* is a halophyte species able to complete its life cycle under exposure to 400 mM of NaCl [52]. However, we demonstrate that the germination process is much more salt-sensitive, with a reduction of almost 60% when exposed to 200 mM of NaCl (Figure 1). An even more drastic reduction in the germination rate in *L. maritima* due to salinity (100 mM NaCl) was reported by Zammali et al. [43]. Based on this information, we selected 150 mM NaCl as the suitable experimental salt concentration to test the effects of PGPB inoculation on this critical stage of plant establishment under saline conditions. Although *S. alba* is not a halophyte, NaCl exposure up to 150 mM decreased its seed germination rate to a similar extent as that observed for *L. maritima* (Figure 1). Thus, 150 mM of NaCl was also selected for *S. alba* to continue the experiments. *Brassica napus* has been reported as a crop with a certain salt tolerance, where a screening of 549 *B. napus* inbred lines with different genetic backgrounds determined that 200 mM NaCl was the concentration at which the germination rate decreased up to 50% in almost all the lines evaluated [53]. This contrasts with our *B. napus* biotype, which was the most salt-sensitive species and showed significant reductions in the germination rate even at 50 mM NaCl (Figure 1). However, as 75 mM NaCl caused a decrease in germination of around 50% in *B. napus*, this concentration was selected for further experiments in this species.

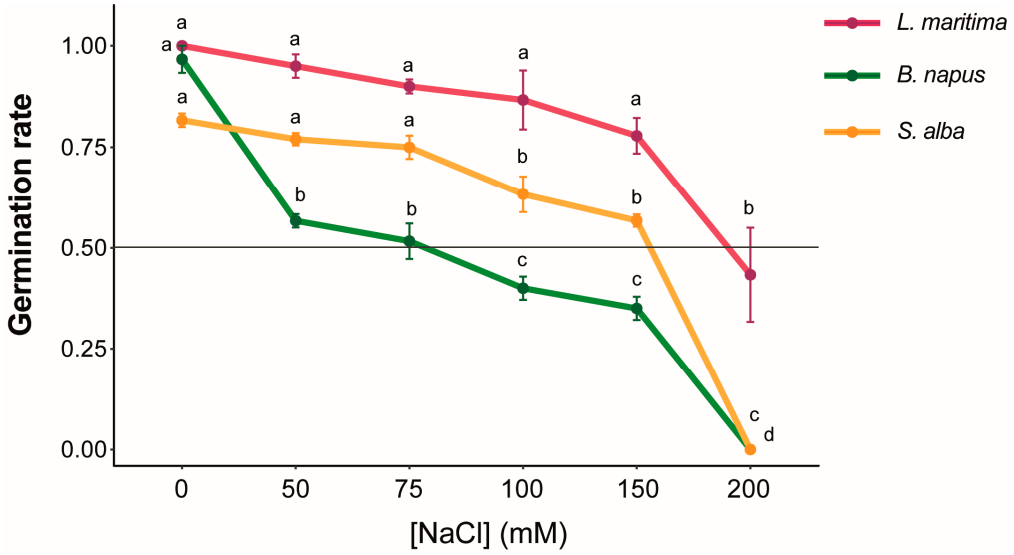

Figure 1. Effects of salt on the germination of Brassicaceae species. Germination rate of *L. maritima* (maroon), *B. napus* (green), and *S. alba* (yellow) sown on MS plates with increasing concentrations of NaCl. Different letters indicate significant differences among NaCl treatments (Duncan test, $p < 0.05$).

The plant microbiome is shaped by interactions among the host, the environment, and the associated microorganisms. Crop domestication has led to genetic changes in many traits involved in the recruitment of host-specific root microbiota, resulting in a microbiome biodiversity loss in comparison to wild relatives [54,55]. The use of PGPB inoculants can compensate for this deficit by becoming a promising alternative to enhance crops performance and yield under changing environments. Therefore, we selected two PGPB strains to evaluate whether their inoculation on seeds of different Brassicaceae species benefits plants cultivated under saline conditions. The PGPB strains used in this study

were previously selected in an in vitro assay due to their ability to solubilize phosphate, produce siderophores and IAA-like molecules, and fix atmospheric nitrogen, along with their tolerance to increasing salt concentrations (up to 300 mM) [44]. Firstly, an adhesion test was performed to determine how many colonies were attached to the seeds of the three species after the inoculation process. The results showed that the cfu/seed of the T7 and E1 strains were reduced by two and four orders of magnitude, respectively, compared to the initial bacterial culture (10^9 cfu/mL). This reduction was expected, as similar results were observed in soybean and peanut seeds inoculated with different *Bradyrhizobium* sp. strains [46,56]. However, a significant reduction was clearly detected when comparing both strains in the same plant species, with T7 the strain being that was strongly attached to the seeds compared to E1 (Table 1). This indicates the specific capacity of each microorganism to attach to seeds.

Table 1. Bacterial adhesion on Brassicaceae seeds. Data represent the mean ± SE (n = 3). Different letters on each column indicate significant differences between PGPB strains according to Duncan test ($p < 0.05$).

	L. maritima	***B. napus***	***S. alba***
		(cfu/seed)	
T7	$1.71 \times 10^7 \pm 1.48 \times 10^5$ (a)	$1.37 \times 10^7 \pm 1.10 \times 10^6$ (a)	$2.08 \times 10^7 \pm 9.27 \times 10^5$ (a)
E1	$3.96 \times 10^5 \pm 1.35 \times 10^5$ (b)	$2.71 \times 10^5 \pm 2.90 \times 10^5$ (b)	$9.16 \times 10^5 \pm 6.00 \times 10^4$ (b)

After inoculation, seeds were sown in MS plates with 0 mM NaCl (control plates), 75 mM, or 150 mM NaCl (treatment plates). A third group of plants was maintained without PGPB inoculation, referred to as the N/I group. Seed inoculation with either T7 or E1 induced an amelioration of the germination rate in *S. alba*, while no significant differences were observed in *L. maritima* or *B. napus* (Figure 2A). Although plants can control seed germination depending on environmental conditions, under salinity, the mechanisms that regulate seed germination may differ in distinct Brassicaceae species [57]. The role of PGPB in seed germination is linked to the production of hormones such as IAA or gibberellins [58]. The PGPB used in this study are producers of IAA-like molecules, which may be one of the mechanisms underlying the positive effects of inoculation [44].

As in vitro trials may not represent the performance of plants from an agricultural point of view, we evaluated the effects of PGPB T7 and E1 on plants growing in saline soil. Inoculated seeds were germinated in sterile soil watered with 75 mM NaCl to simulate the salinity levels that are frequently reached in Mediterranean cropland [17,58]. *Lobularia maritima* seeds inoculated with either T7 or E1 showed better germination and seedling establishment rates in the control soil, while only T7 enhanced these parameters under salt conditions (Figure 2B). In *B. napus*, T7 increased the germination and seedling emergence under control conditions. Surprisingly, the use of T7- or E1-inoculated seeds in the saline soil led to a decrease in the germination rate of *B. napus* compared to the N/I seeds. However, all the germinated inoculated seeds were able to be properly established under this stress (Figure 2B). *Sinapis alba* germination was not altered when either T7 or E1 strains were inoculated, showing a milder effect compared to the in-vitro assay (Figure 2). The influence of PGPB–host specificity on seed germination observed in our results is consistent with findings in *Acacia senegal*, where seed inoculation with *Sinorhizobium saheli* (PC-6) enhanced the germination capacity of the CAZRI 35A genotype, but decreased it in CAZRI 113AS [59].

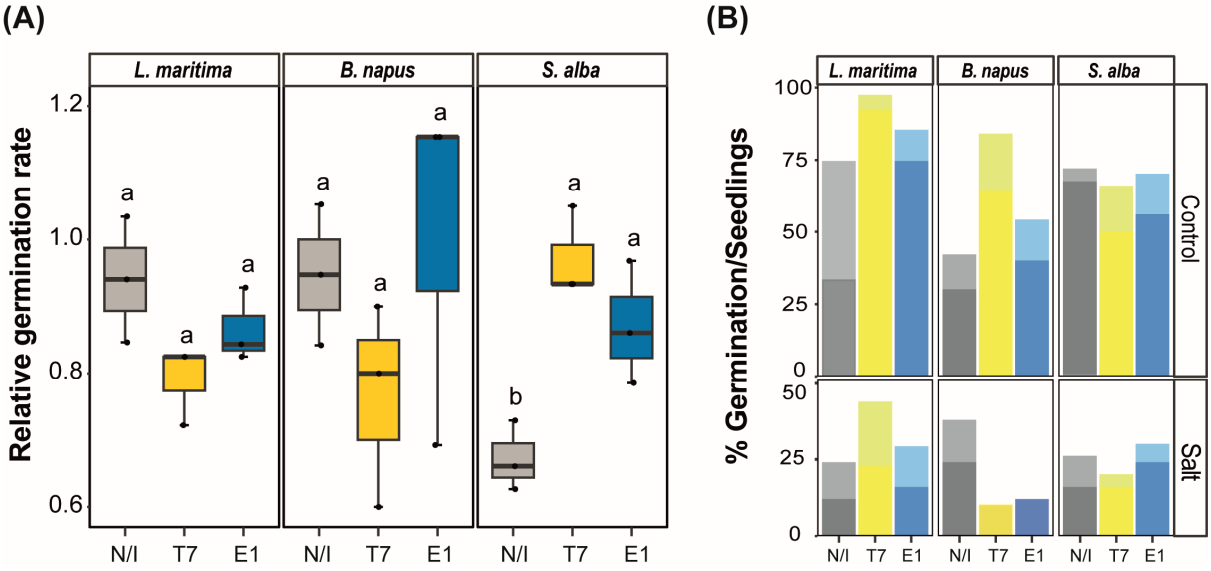

Figure 2. Effects of PGPB seed inoculation on the germination of Brassicaceae species exposed to salinity. (**A**) Relative germination rate ($Germination_{Salt}/Germination_{Control}$) of *L. maritima*, *B. napus*, and *S. alba* seeds N/I (grey) or inoculated with T7 (yellow) or E1 (blue) sown in MS plates with 0 or 75 mM/150 mM NaCl. Different letters indicate significant differences among inoculation conditions (Duncan test, $p < 0.05$). (**B**) Percentage of germinated seeds (light color) and established seedlings (dark color) of *L. maritima*, *B. napus*, and *S. alba* seeds N/I (grey) or inoculated with T7 (yellow) or E1 (blue) sown in sterile substrate irrigated with water containing 0 or 75 mM NaCl.

3.2. Seedling Establishment of Brassica Seeds Inoculated with PGPB Under Saline Soil Conditions

Salinity stress not only affects seed germination, but also impairs growth post-emergence, causing reduced development or, in some cases, plant death [60]. To evaluate how salinity and inoculation conditions alter seedling establishment, we measured the shoot lengths of all the emerged plants at three time points over the first 15 days. All the species were affected by salinity stress, as evidenced by the general lower shoot length of all plants growing under salinity (Figure 3). Additionally, the three species also exhibited delayed growth when exposed to salinity, with a slower increase in shoot length from day 5 to 10 than from day 10 to 15 (Figure 3). The period until seedlings are completely established is critical, as it is a highly sensitive stage to abiotic stress. Salt stress induces ion toxicity, which inhibits enzyme activity, thereby causing alterations in storage protein hydrolysis and starch remobilization [61]. This situation leads to reduced photosynthetic pigment content and limited cotyledon growth, resulting in delayed seedling establishment [62]. This response can be reversed by PGPB inoculation [63–65]. Our results indicate that inoculation with the T7 strain significantly promoted the growth of *L. maritima* and *B. napus* under control conditions (Figure 3A,B). However, under saline conditions, a significant increase in shoot length was observed only when *B. napus* was inoculated with the E1 strain and when *S. alba* was inoculated with T7 (Figure 3B,C). Notably, the inoculation of *L. maritima* with E1 had no positive effect (Figure 3A), while *S. alba* plants inoculated with E1 outperformed the N/I ones under both conditions, in spite of not presenting significant differences (Figure 3C). Similar studies have shown that growth promotion of broccoli, mallow, and canola plants cultivated under saline or saline–alkaline stress depend on specific PGPB–plant interactions, demonstrating that not all beneficial microorganisms impact plant species in the same way [66,67]. In summary, these results denote that, under control conditions, our PGPB have a positive impact on seedling development. However, under saline conditions, both *S. alba* and *B. napus* experienced significant accelerated growth, but with high specificity for a PGPB strain (Figure 3).

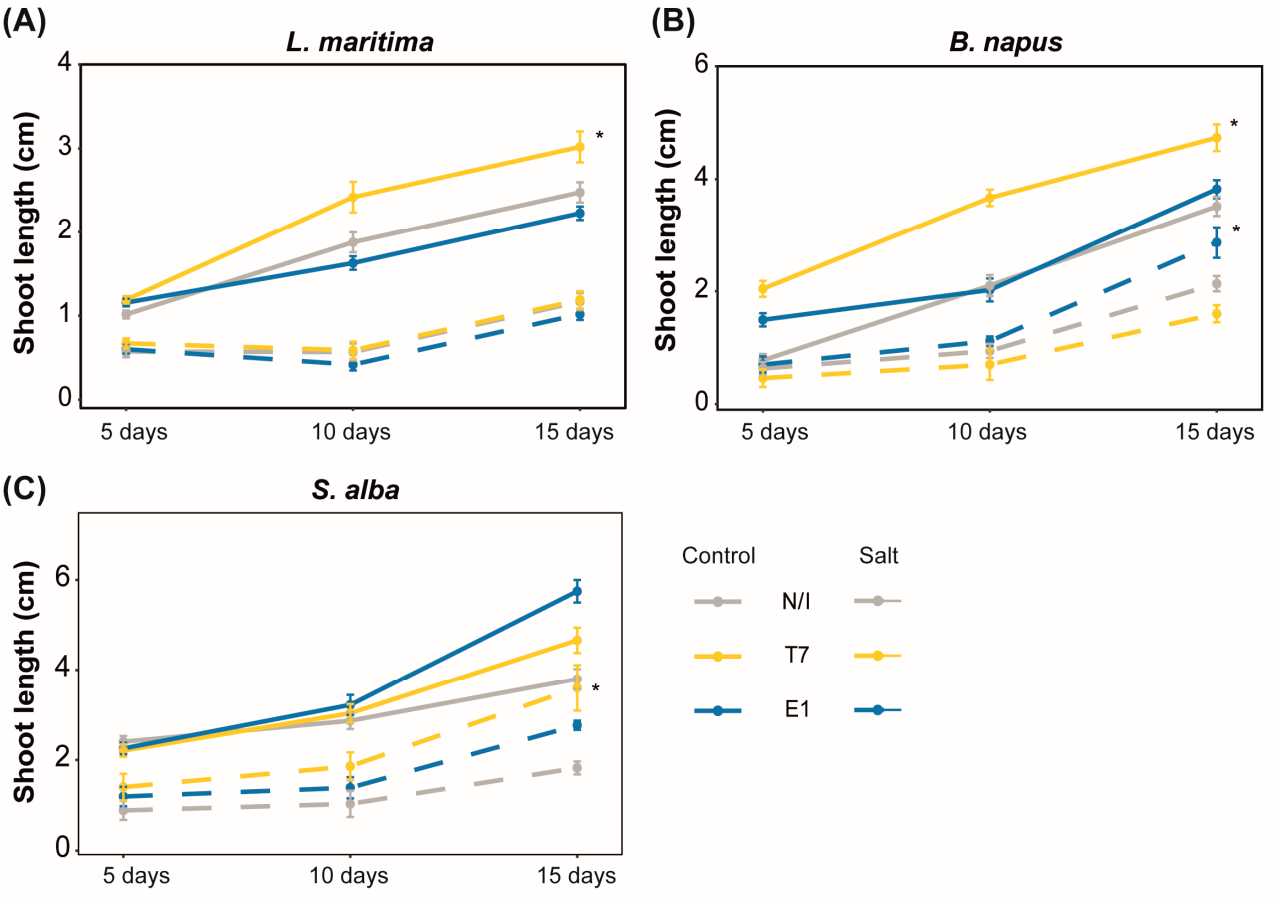

Figure 3. Seedling growth of PGPB-inoculated Brassicaceae species exposed to salinity. Mean ± SE of shoot length (cm) of (**A**) *L. maritima*, (**B**) *B. napus*, and (**C**) *S. alba* seedlings N/I (grey) or inoculated with T7 (yellow) or E1 (blue) cultivated in sterile substrate irrigated with water containing 0 (solid lines) or 75 mM NaCl (dashed lines). Asterisks indicate significant differences among inoculation conditions (MANOVA repeated measures, $p < 0.05$).

3.3. Seedling Growth and Root Architecture of Brassicaceae Species Inoculated with PGPB Under Saline Soil Conditions

It is well known that salinity stress reduces cell division activity and elongation, causing alterations in the root system architecture and a decrease in the growth of the primary root [39,68]. Our results are consistent with these findings, as we observed a decrease in root length in all three species cultivated under salinity, independently of the inoculation condition (Figure 4A). West et al. [69] reported that salinity stress causes quick meristem cell divisions to stop, producing a reduction in the size of the mature cells and leading to a shorter meristem. Focusing specifically on each species, the inoculation with T7 resulted in significant outcomes for both *L. maritima* and *S. alba*, which exhibited higher root length compared to the N/I under both treatments (Figure 4A). Regarding root volume, a significant decrease under salt conditions was also observed in *B. napus* and *S. alba*, while no differences were detected in *L. maritima* (Figure 4B). Reductions in the root volume of glycophytes under saline stress has previously been documented [70,71]. Contrastingly, studies with halophytes have only reported differences in root length [72], supporting our findings in *L. maritima*.

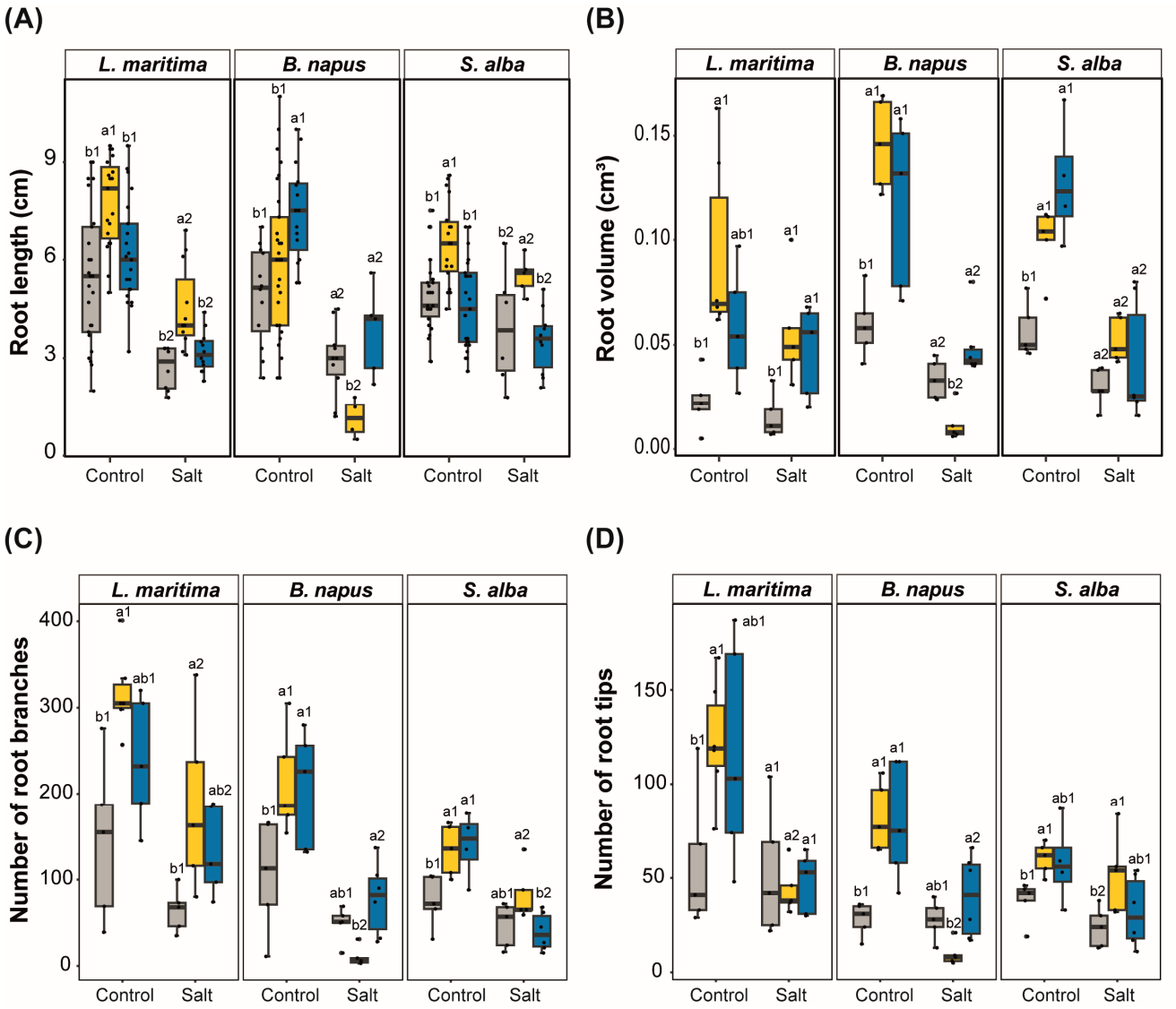

Figure 4. Root growth and architecture of PGPB-inoculated Brassicaceae species exposed to salinity. Mean ± SE of (**A**) root length (cm), (**B**) root volume (cm^3), (**C**) number of root branches, and (**D**) number of root tips of *L. maritima*, *B. napus*, and *S. alba* seedlings N/I (grey) or inoculated with T7 (yellow) or E1 (blue) cultivated in sterile substrate irrigated with water containing 0 or 75 mM NaCl. Different letters indicate significant differences among inoculation conditions (Duncan test, $p < 0.05$), while different numbers indicate significant differences among salt treatments (*t*-test, $p < 0.05$).

To evaluate the alterations in the root system architecture caused by salt stress, changes in the number of root branches and tips were also analyzed. Both bacterial inoculations resulted in significant increases in root volume, as well as the number of branches and tips under control conditions in all species, except for the E1 strain in *L. maritima* (Figure 4B–D). Conversely, under saline conditions, PGPB significantly increased the root volume only in *L. maritima* seedlings (Figure 4B), and the inoculation with T7 raised the number of root tips in *S. alba* (Figure 4D). These analyses of root morphological traits indicate that bacterial inoculations can significantly alter root architecture under control conditions, but the effects are less remarkable under salt stress.

A characteristic effect of PGPB is the promotion of a more branched root system potentially driven by the stimulation of lateral root formation [73–75]. In plants, this process is highly influenced by hormone signaling pathways, especially auxins, which play different roles in triggering lateral root initiation [76]. Liu et al. [77] reported that the exogenous application of this hormone to *A. thaliana* increases both the root meristem length and the number of meristematic cells when exposed to 100 mM NaCl. Indeed, the inoculation with an IAA -producing bacteria revealed higher levels of this hormone in the root tissue of *Sedum alfredii*, leading to an increase in the root length [75]. In addition, PGPB that have ACC deaminase enzymes can increase lateral roots through the regulation of ethylene and IAA [78,79]. Previous findings showed that T7 and E1 strains produce high levels of IAA under both control and salt conditions, and they also contain the enzyme ACC deaminase [44], making them suitable candidates for promoting root branching and enhancing the whole root system's architecture.

Regarding aerial analyses, shoot biomass was reduced in all species when plants were grown under salt stress, but, as observed previously, the responses differed depending on each PGPB–plant species interaction (Figure 5). Seedlings of *L. maritima* inoculated with T7 showed greater aerial growth compared to N/I ones in both control and salt stress conditions, whereas E1 only promoted growth under the control condition (Figure 5A). However, the salinity tolerance index of all inoculated *L. maritima* was significantly higher compared to N/I plants (Figure 5C). Surprisingly, the leaf Na^+ concentration was also higher in the inoculated plants (Figure 5D), but the levels detected did not reach toxic concentrations for a halophyte such as *L. maritima* [80]. A similar effect was observed in *B. napus* control plants. Nevertheless, under salinity treatment, T7-inoculated *B. napus* plants showed reductions in aerial biomass and low salinity tolerance indices compared to N/I, while no differences were observed with E1 inoculation (Figure 5A,C). Contrastingly, *B. napus* plants inoculated with T7 were the only ones able to restrict Na^+ translocation (Figure 5D). It is well known that salt tolerance strategies can involve high physiological costs [55]; thus, T7 inoculation could promote the production of osmolytes and antioxidants that improve plant survival, but cause a growth penalty. In the case of *S. alba*, no inoculation effects were observed under control conditions, but seedlings inoculated with T7 showed greater biomass at 75 mM NaCl (Figure 5A). Although these results were not significant, *S. alba* plants inoculated with T7 exhibited higher salinity tolerance indices and lower Na:K ratios than the N/I (Figure 5C,D). Saghafi et al. [41] showed that ACC-deaminase-producing halotolerant bacteria (like T7) promoted K^+ uptake in canola plants cultivated under salinity, enhancing plant growth and mitigating the salt stress effects. We hypothesise that the enhancement of biomass and leaf Na^+ and K^+ concentrations could be related to (i) the better development of the root system observed in the inoculated Brassicaceae plants (Figure 4) and (ii) the ability of the bacteria to activate plant salinity tolerance strategies mediated by their PGP mechanisms.

Growth promotion by PGPB depends on rather specific plant–bacteria cross talk [69]. Previous studies showed that the effect of PGPB on germination and seedling emergence might be related to how those strains are initially perceived by different plant species [81,82]. For instance, while onion seedlings inoculated with a *Pantoea* sp. strain showed greater hypocotyl and radicle growth, the inoculation of this microorganism in pak choy and sweet pepper diminished plant growth [83]. Such specificity is supported by our results, where, under salt stress, T7 inoculation in *B. napus* caused biomass reduction, while an enhancement was observed in *S. alba* (Figure 5A,B). In addition, PGPB-induced growth stimulation is mainly due to either enhanced mobilization of mineral nutrients, the production of phytohormones by the bacteria, or both. Growth inhibition, as observed here in *B. napus*, has only occasionally been reported and has been attributed to a high supply of auxin [16]. This hormone has inhibitory effects at high concentrations.

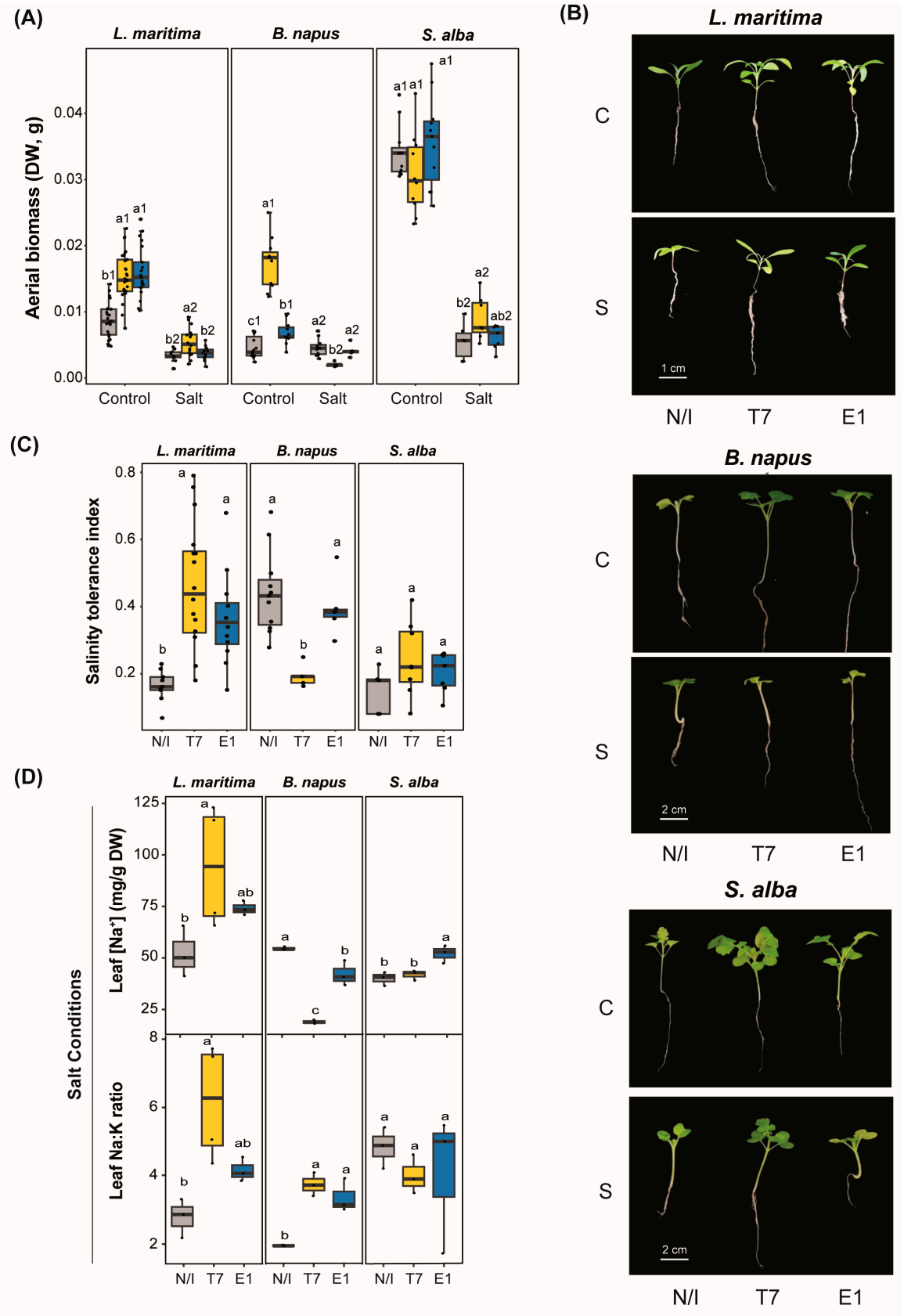

Figure 5. Salinity tolerance of PGPB-inoculated Brassicaceae species. (**A**) Mean ± SE of aerial biomass (shoot dry weight, g), (**B**) representative pictures, (**C**) salinity tolerance index, and (**D**) leaf Na^+ concentration (mg/g DW) and Na:K ratio of *L. maritima*, *B. napus*, and *S. alba* seedlings N/I (grey) or

inoculated with T7 (yellow) or E1 (blue) cultivated in sterile substrate irrigated with water containing 0 or 75 mM NaCl. Different letters indicate significant differences among inoculation conditions (Duncan test, $p < 0.05$), while different numbers indicate significant differences among salt treatments (*t*-test, $p < 0.05$).

3.4. Detection of Oxidative Damage in Brassica Seedlings Inoculated with PGPB Under Salt Conditions

The oxidative burst caused by the overproduction or accumulation of ROS (e.g., O_2^-, H_2O_2) under salinity stress is a common response observed in various plant species, including cereals, legumes, and brassicas [84–87]. Our results partially agree with these findings, since all N/I tested plants except *B. napus* increased ROS levels under salinity (Figure 6A). PGPB may palliate this stress-induced ROS production [88]. However, early ROS production can also be activated during the interaction of plants with either beneficial or pathogenic microorganisms. PGPB-induced ROS can act as signalling molecules, triggering an appropriate acclimation response under unfavourable growth conditions or, alternatively, as toxic radicals that damage vital molecules [89]. In our study, the inoculation of T7 in *L. maritima*, *B. napus*, and *S. alba* exposed to salinity significantly enhanced peroxide levels compared to controls (Figure 6A). The results observed with E1 inoculation differed among plant species. In *L. maritima*, the concentration of peroxides was maintained as under control conditions, while in *S. alba*, a significant increase was detected, and in *B. napus*, peroxide concentrations were reduced. Under saline conditions, no differences were observed between inoculated and N/I plants of *L. maritima* and *S. alba*, indicating that the PGPB did not modify the oxidative burst imposed by the treatment. Nonetheless, in *B. napus* plants inoculated with T7, a significant increase in the H_2O_2 concentration was observed compared to N/I plants, showing a distinctive response in the plant–microbe interaction. The impact of PGPB on plants under abiotic stress has been extensively studied, with most research focusing on the PGP mechanisms that stimulate plant antioxidant activity [15,90]. However, studies specifically examining ROS production in brassica under salinity and inoculated with PGPB remain limited. In contrast to our results, Neshat et al. [32] observed that inoculation of canola with the PGPR *Enterobacter* sp. S16-3 or *Pseudomonas* sp. C16-20 significantly reduced the level of salinity-induced hydrogen peroxide.

To further characterize the differential oxidative behavior of our experimental plants, we determined the lipid peroxidation as a well-known stress marker. Salinity induced lipid peroxidation in *L. maritima* and *S. alba*, indicating impairment of cell membrane integrity (Figure 6). However, it is known that inoculation of PGPB can reduce oxidative stress, activating the antioxidant system by regulating the expression of genes related to ROS scavengers in plants [91]. In our study, when PGPB were inoculated in brassica plants, a significantly reduction in the variable under the treatment compared to N/I was observed, except for *B. napus*. In agreement with our results, it has been demonstrated that inoculation of maize with *P. pseudoalcaligenes* reduces the MDA content [92]. Also, in soybean, a reduction in the oxidative stress was detected when inoculated with a *Bacillus* strain [93]. In addition, white clover showed a reduction in lipid peroxidation growing under salt when a *B. subtilis* (GB03) strain was inoculated. Similar behaviour was demonstrated in wheat inoculated with *B. megaterium*, maize with *Kocuria rhizophila* Y1, and canola with *E. cloacae* HSNJ4 subjected to abiotic stress [64,94,95]. In our work, contrasting behaviour was observed in *B. napus*; PGPB inoculation with either T7 or E1 induced severe peroxidation of lipids compared to (N/I) plants (Figure 6B). A similar result was reported in a study with canola where plants inoculated with *Pseudomonas* sp. C16-20 maintained higher levels of lipid peroxidation than N/I plants or those inoculated with *Enterobacter* sp. S16-3 [32]. Our findings in *B. napus* reflect that the establishment between the plant and the PGPB results in an increment in the oxidative damage, probably linked with the high level of H_2O_2 observed.

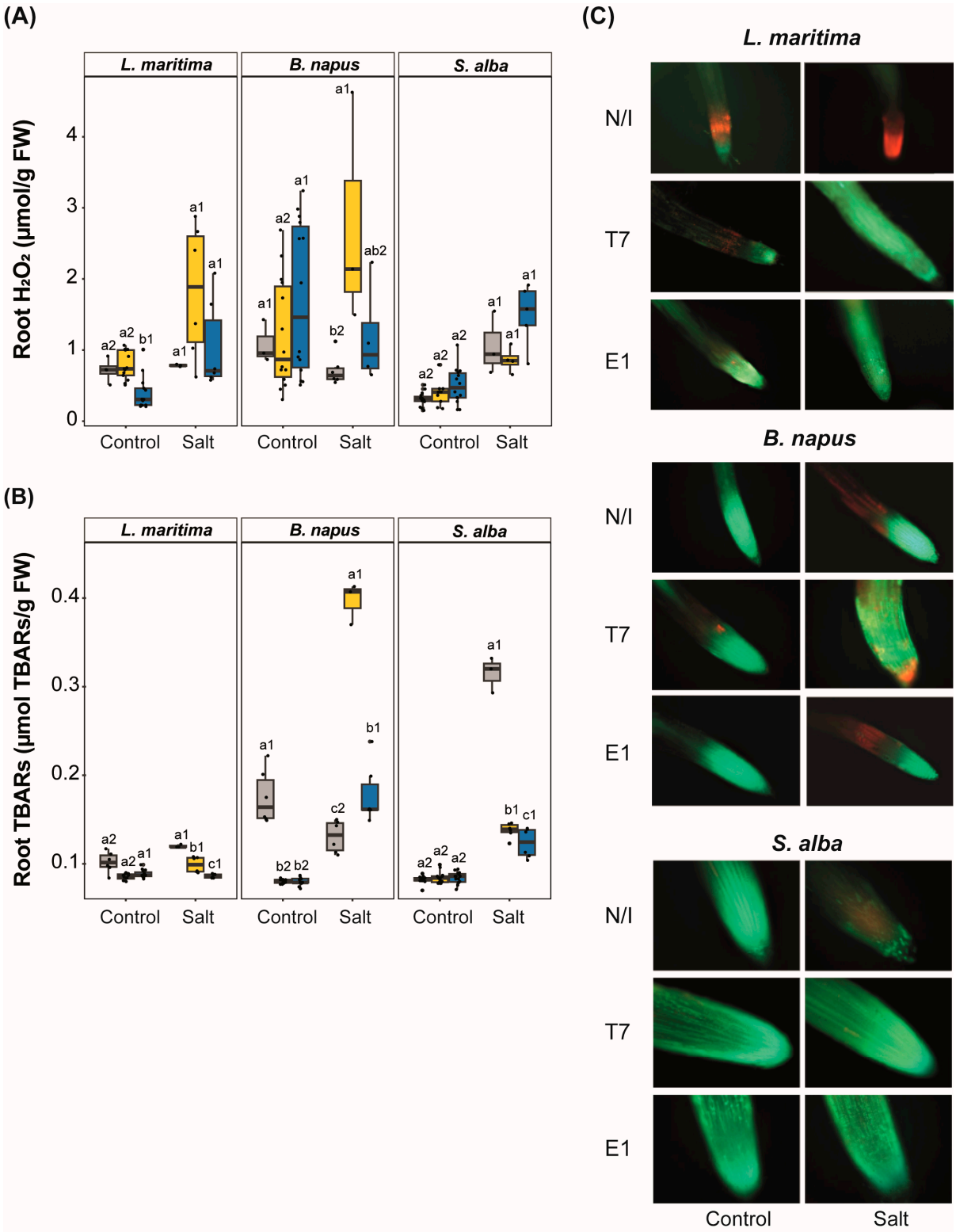

Figure 6. Oxidative damage in roots of PGPB-inoculated Brassicaceae species exposed to salinity. Mean ± SE of (**A**) root hydrogen peroxide concentration (µmol H_2O_2/g FW), (**B**) root lipid peroxidation (µmol TBARs/g FW), and (**C**) FDA-PI-stained roots (10× magnification) of *L. maritima*, *B. napus*, and *S. alba* seedlings N/I (grey) or inoculated with T7 (yellow) or E1 (blue) cultivated in sterile substrate irrigated with water containing 0 or 75 mM NaCl. Different letters indicate significant differences among inoculation conditions (Duncan test, $p < 0.05$), while different numbers indicate significant differences between salt treatments (*t*-test, $p < 0.05$).

Additionally, we performed vital staining with PI and FDA, a cytology technique to determine cell viability, which serves as an important tool for evaluating lethal salinity effects on plant root cells. This technique reveals cell death through the visible red staining that occurs when the dye enters cells with compromised membrane integrity [13]. The results showed that all N/I plants exposed to salinity induced PCD in the apex or in the elongation root zone compared to the control, indicating the severe effects of the treatment (Figure 6C). PGPB inoculation with either T7 or E1 strains reduced the salt-induced damage in both *L. maritima* and *S. alba*. However, in *B. napus*, PCD was maintained or even increased (Figure 6C). Thus, in contrast to the *L. maritima* and *S. alba* results, the interaction between our PGPB strains and *B. napus* under saline treatment did not ameliorate the stress impaired by salinity, but rather exacerbated it. The differential response of *B. napus* may be related to the considerably high number of AUX/IAA genes in this species in comparison to other Brassicaceae [96]. Excess IAA may limit the division growth rate. Furthermore, the high energy demand to reduce Na^+ translocation, as observed previously (Figure 5), may contribute to the inhibitory effect. These findings support the view that, at an early stage of growth, a specific strain–plant–treatment interaction occurs, leading to a differential oxidative response.

4. Conclusions

Due to domestication, crop species such as the valuable Brassicaceae selected for this study may have experienced a loss of biodiversity in their own microbiome, becoming more susceptible to the consequences of climate change. It is well known that inoculation with PGPB, especially native strains, could counteract the negative effects of abiotic stresses such as salinity. Indeed, the results of this study demonstrate that plant–microorganism interactions are tightly regulated, balancing plant ROS induction with the microorganisms' ability to modulate this oxidative response. This balance is essential for maintaining a positive interaction that enhances plant growth under stress conditions. Specifically, in *L. maritima* and *S. alba*, the PGPB–plant interaction resulting from the inoculation of selected beneficial microorganisms such as the *Pantoea* sp. T7 mitigates the adverse effects of salinity. This is evidenced by improvements in dry weight, root length, root volume, and the number of root branches. The positive effects of the bacterial inoculation could be observed later, at the seedling stage of growth, rather than on germination rates, indicating that a time-dependent response is needed to achieve positive results in the tested brassica plants exposed to salinity. The contrasting results in *B. napus*, where the PGPB inoculation enhanced salinity-induced oxidative stress, highlight the specificity of the plant–bacterial interaction. This specificity is a key characteristic which must be carefully considered in the design of strategies for the practical use of PGPB in crop protection against salinity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14122184/s1, Dataset S1: Statistical results of the manuscript data analyses. Figure S1. Scan pictures of Brassicaceae seedlings used for WinRhizo analyses. Figure S2. Relative germination rate of Brassicaceae seeds sown with different concentrations of salt. Figure S3. Leaf K^+ concentration (mg/g DW) of Brassicaceae seedlings inoculated with PGPB and cultivated under salt stress conditions.

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