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Coastal Almond-Leaved Pear (*Pyrus spinosa*) Seedlings' Responses to Saline Stress Alleviated by Formulated L-Methionine and Bacterial Exogenous Soil Application

Helen Kalorizou [1,*], Paschalis Giannoulis [2], Stefanos Leontopoulos [3], Charalambos Angelakis [1] and Maria Sorovigka [1]

[1] Department of Agriculture, Faculty of Agricultural Sciences, University of Patras, New Buildings, 30200 Missolonghi, Greece; up1076306@ac.upatras.gr (C.A.); up1076832@ac.upatras.gr (M.S.)
[2] Department of Agrotechnology, Faculty of Agricultural Sciences, University of Thessaly, Geopolis Campus, 41100 Larisa, Greece; pgiannoulis@uth.gr
[3] School of Applied Arts and Sustainable Design, Hellenic Open University, Parodos Aristotelous 18, 26335 Patras, Greece; s_leontopoulos@yahoo.com
* Correspondence: ekalorizou@upatras.gr; Tel.: +30-2631058232

Abstract: Coastal *Pyrus spinosa* seedlings were tested for their developmental, chlorophyll content and antioxidant performance under soil saline conditions where bacterial and l-methionine exogenous treatments were applied as potential saline alleviation stress schemes. Scaling up saline stress, the number of formed lateral shoots was reduced in all treatments. Medium salt stress (75 mM NaCl) demonstrated a rather unified decline in shoot fresh weight values, which became toxic at 100 mM NaCl, with up to 89.1% shoot fresh weight losses, in comparison to unchallenged status. Both exogenous applications increased root/shoot ratio, providing developmental boost for root growth. Total chlorophyll content values (May–July) did not differ among non-stressed plantlets independently of exogenous treatment. All experimental plantlet lines increased their antioxidant activity on scaled up soil NaCl enrichment. No differences in root orientation and their angle frequencies were observed while soil saline exposure took place. In brief, spring–summer exposure of *P. spinosa* plantlets under 100 mM NaCl saline stress can be manageable, achieving higher root/shoot ratio values, upregulating leaf antioxidant activity and optimizing root growth upon bacterial and l-methionine supplementation. However, many of the examined parameters were found to be not extensively different between exogenously treated plantlets and non-supplemented ones, suggesting a potential role of intergenerational and transgenerational stress memory.

Keywords: *Pyrus spinosa*; salinity effect; l-methionine; *Bacillus* sp.; *Azotobacter* sp.

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1. Introduction

Pyrus spinosa (syn. *amydaloformis*; common name: almond-leaved pear) is one of the wild members of *Pyrus* species originated from western and southwestern mountainous areas of China with tremendous evolutionary impact in entrepreneurial orchard farming and fruit production [1,2]. As a common wild habitat, almond-leaved pear forms small isolated populations with high levels of phenotypic plasticity, which are mainly dispersed by birds and mammals in woodland pastures [3,4]. The native profile of *P. spinosa* reflects an extremely drought-resistant plant species with quite flexible physiology in environmental extremes [5]. Agronomically, (a) it easily hybridizes with *P. communis*, *P. elaeagrifolia* and other genus *Pyrus* members, offering novel sets of rootstocks especially resistant to lime chlorosis, (b) it plays a key role in apiculture due to it being rich in amino acid content of nectar and (c) it improves soil carbon storage under agroforestry cultivation schemes [4,6–10]. *P. spinosa* fruit pulp exhibits antibacterial and antifungal activities due to their richness in linoleic and oleic acids; bark extracts of the tree demonstrate antimicrobial and cytotoxic activities against

malignant human cell lines, suggesting a promising spectrum of uses in pharmaceutical science [11,12].

Soil salinity is a time-lapse expansive phenomenon that affects food reserves and rural development around the globe. Advances in orchard irrigation and soil amelioration cannot solely counteract salinity consequences due to the fact that fresh and clean water availability is not guaranteed for many places on earth; physiologically resilient plant material must also be used to overcome fruit production yield losses [13–15]. For perennial plants like trees, soil saline conditions form a complex of induced and acquired memory when intergenerationally and simultaneously exposed to the stress factor [16]. Recorded up to now, non-wild, commercial *P. communis* orchards exhibit salt tolerance with trade-off cost of reduced shoot growth at electrical conductivity values higher than 5.0 dS/m, regardless of quince rootstock genetic differentiations [17]. *Pyrus spinosa* is a plant species resistant to drought and salinity, spatially distributed in coastal sand dunes as part of its native habitat [18]. However, its adaptation mechanisms to saline stress are poorly documented.

Methionine is an essential amino acid affecting plant nutrition, plant defense to stresses and immunity; it is transcriptionally interdependent to threonine and isoleucine availability when abiotic stress conditions such as drought and salinity occur [19–21]. Exogenous methionine application in horticultural species under saline stress conditions improves growth, fresh biomass and fruit yield, whereas jointly applied with l-phenylalanine affects positively free amino acid, carotenoid and total carbohydrate contents. This resilient-to-salinity, enriched methionine profile is also coupled with increased proline existence, higher plasma membrane stability and a plethora of osmolyte substrates present [22]. Endogenously, methionine leads to synthesis of (a) *S*-methymethionine (its mobile and storage forms), (b) *S*-adenosylmethionine, which regulates ethylene, polyamines and biotin and (c) precursor molecules for secondary metabolite production with osmoprotectant properties like 3-dimethyl-sulfoniopropionate [23]. Methionine oxidation is capable to modulate phosphatase and kinase activities, which are involved in cellular signaling under saline stress conditions; this process is fully reversible via methionine sulfoxide reductase enzymatic activity [24,25].

Soil-beneficial *Bacillus* bacterial communities were found to optimize tree nutritional physiology and positively affect plant hormonal status, advancing resilience towards biotic and abiotic stress factors including salinity [26,27]. Furthermore, members of bacterial genus *Azotobacter* provide rhizosphere counter-saline osmoprotectants like deaminases, salicyclic acid, proline and exopolysaccharides, attempting to restore unchallenged status for plants and farming activities in arid areas [28].

Phenolic content of *P. spinosa* consists of a front-line, interdisciplinary field in agriculture, medicine and pharmaceutical sciences [29–32]. *P. spinosa* leaves are richer in total phenolics in comparison to seeds and fruits where gallic acid, chlorogenic acid, rutin, coumaric acid, quercetin, apigenin and arbutin are abundant and dominant [33,34]. Hybrid rootstocks of *P. spinosa* exudate phenols in the rhizosphere in order to become tolerant to soil-induced iron chlorosis [35]. However, investigation of bark total phenolics and DPPH radicals of *P. spinosa* reveal lower levels of response in comparison to *Pyrus communis* subsp. *pyraster* [31].

Herein, we examine *P. spinosa* seedlings for their growth resilience against soil salt stress, measuring changes in their shoot and root growth characteristics as well as leaf parameters (chlorophyll content, total phenolics, antioxidant activity) by exploring alternative alleviation stress techniques, administrating soil-formulated l-methionine biostimulant and bacterial inoculums of *Bacillus* and *Azotobacter* species.

2. Materials and Methods

2.1. Plant Materials

Mature fruits from wild pear trees (*Pyrus spinosa*) were collected from the coastal area of Central Greece (38°23′19.18″ N; 22°22′23.18″ E). Seeds were separated from fruit pomace manually with a knife and were washed several times with distilled water for pomace

residual removal. In order to break seed dormancy, seeds were sterilized by sinking in 2% sodium hypochlorite solution for 5 min and then were placed in 500 ppm gibberellic acid (GA_3) solution for 12 h. Finally, seeds were transferred to sterilized plastic box with wet cotton as a substrate and kept in low temperature (4 °C). After 30 days at cold storage, seeds were sowed in trays with substrate mixture of peat/perlite (1:2) and kept in greenhouse for germination and growth. For experimental purposes, sixty uniform 3-month-old plantlets were selected and transplanted to 1 L pots with the same substrate.

2.2. Biochemical and Microbial Saline Stress Alleviation Schemes

Formulated biostimulant rich in l-methionine (NPK 5-20-0+5% l-methionine; commercial name PHYTOAMINO®-PN produced by Karvelas S.A., Agrinio, Greece) and plant growth promoting bacteria (NPK 0.6-1.2-3+0.3% CaO, 0.1% MgO, 0.1% S; *Bacillus subtilis*, *Bacillus pumillus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Azotobacter* sp.; commercial name RHI-ZOBAC produced by Humofert S.A., Metamorfosi, Athens, Greece) at 1×10^{11} cfu/L were applied in separate cultivation lines. Applications took place after seedling transplantation to 1 L pots once every 15 days for 3 months with standardized solutions of 2% and 1%, respectively.

2.3. Saline Exposure

Four levels of salinity stress were selected to challenge *P. spinosa* plantlets. A total of 200 mL of NaCl water solution in the following concentrations 0 mM, 50 mM, 75 mM and 100 mM were used to stress plantlets as irrigation regime, once every two days for 3 months (end of April–end of July). Months April to July were selected for experimentation in order to deliver robust data for (a) saline effect on *P. spinosa* seedlings, (b) *P. spinosa* plantlet limits of phenotypic and physiological plasticity in presence of alleviation schemes and (c) efficacy of the above applied schemes.

2.4. Measurement of Plant Growth Parameters

Shoot length from soil to shoot tip was recorded every 30 days coupled with calculation of growth increment percentage. At the end of experimental period, 3 months later, shoots separated from roots, both of them were washed carefully and their fresh weight was measured. The number and length of secondary shoots were also analyzed. Morphological parameters that reflect quantitative characteristics of root size and architecture, like median number of roots, total root length, root diameter, projected root volume, root orientation, root angle frequencies and root length per root thickness diameter, were determined using RhizoVision Explorer v2.0.3 open-source software for image analysis [36]. Root and shoot biomasses were calculated after shade drying until their weights remained constant.

2.5. Measurement of Total Chlorophyll Content

The total chlorophyll content was determined during the experimental period using the CCM-200 Plus (OPTI-SCIENCES, Hudson, NH, USA) Chlorophyll Content Meter. Measurements were taken every 30 days. Five new fully expanded uppermost leaves per plant were chosen to be examined for their chlorophyll content, and changes were recorded under different levels of saline stress exposure and types of exogenous supplementations. Measurements taken every 30 days from end of April to end of July. From each leaf, five SPAD records were taken from the middle of leaf lamina so as to calculate the mean SPAD value per leaf.

2.6. Total Phenolic Content and Antioxidant Activity

Leaf extract: At the end of saline exposure period, plant shoots of all treatments were harvested and left to dry under natural conditions at room temperature. Leaves separated from shoots and grounded in grinding mill (KINEMATICA, POLYMIX PX-MFC 90 D Blade Grinding Mill, Malters, LU, Switzerland). A total of 1 g of grounded leaves per sample added in falcon tube with 20 mL of ethanol (80%) and left in shaker stirrer (170 rpm) for

24 h at room temperature. The extract was delivered by solvent filtration with Whatman filter paper No 1.

Determination of total phenolic content: The Folin–Ciocalteu assay was used to determine total phenolic content (TPC) according to Singleton and Rossi (1965) [37] with some modifications. Briefly, 1 mL of leaf extract was mixed with 9 mL distilled water and 0.5 mL of Folin–Ciocalteu reagent (10% v/v FC reagent to distilled water). After 3 min, 1.5 mL aqueous Na_2CO_3 solution (20% w/v) was added, and the mixture was kept for 60 min in dark at room temperature. The absorbance was measured at 760 nm using a UV–VIS spectrophotometer (Shimadzu UV 1900i, Kyoto, Japan). Gallic acid (GAE) used as reference standard. A calibration curve was prepared using the absorptions of 5 different concentrations of gallic acid (10, 20, 30, 40 and 50 ppm). The linear equation obtained from gallic acid standard curve (y = 0.0317x + 0.0336, R^2 = 0.9923) was used for sample TPC quantification. The results were expressed as mg of gallic acid equivalent per gram dry weight of leaves (mg GAE/g d.w.).

Determination of antioxidant activity: Antioxidants of leaf extracts were accessed by DPPH radical scavenging assay (Brand-Williams et al., 1995) [38]. An aliquot (0.1 mL) of leaf extract was added to 3.9 mL of DPPH ethanolic solution (0.06 mM). After a 30 min incubation period in dark at room temperature, absorbance was measured at 517 nm using a UV–VIS spectrophotometer (Shimadzu UV 1900i). Control consists of 3.9 mL of DPPH solution where 0.1 mL of ethanol (80%) was added. The free radical scavenging activity of samples was calculated using the following equation [39]:

$$\% \text{ antioxidant activity} = [(A0 - A1)/A0] \times 100$$

where A0 is the control absorbance, and A1 is the absorbance of sample.

2.7. Statistical Analysis

A factorial completely randomized design with 8 treatments—4 saline level and 2 alleviation schemes (amino acid and microbial)—was used in this study. A minimum of five replicates were used for each value, or otherwise, it was stated.

Data were analyzed using the 95% confidence limits overlap protocol of Sokal and Rohlf (1969) [40]. Table and graphic data were presented as means ± standard error of the mean. An α level of 0.05 was chosen. Prism 8.0 (GraphPad) was used for data analysis.

3. Results

3.1. Number of Lateral Shoots and Fresh Weight of Shoots and Roots

Number of lateral shoots formed in *P. spinosa* seedlings declined upon salt presence independently of exogenous amino acid and microbial support. Soil application of l-methionine biostimulant and microbial complex did not favor lateral shoot formation; however, these applications at 100 mM NaCl stress conditions appeared to force seedlings into a tentative, stress-related regenerative developmental process. Seedlings stressed up to 50 mM NaCl led to 36–82% losses in lateral shoot formation, especially when amino acid or bacterial soil enrichment took place (Table 1).

The highest values of shoot fresh weights were demonstrated in non-stressed *P. spinosa* seedling with no soil exogenous treatment. L-methionine and microbial applications in non-saline soil environments reduced the biomass significantly by 14.9% and 41.0%, respectively. Salt stress presence of NaCl 50 mM suppressed shoot fresh weights in all experimental lines, with lower value data to be recorded for control plantlets (53.35%) followed by l-methionine biostimulant soil application (44.52%). Bacterial-based soil enrichment did not statistically affect shoot fresh weights for the same level of salt stress (NaCl 50 mM). A medium level of salt stress (75 mM NaCl) demonstrated a rather unified decline in shoot fresh weights among all applied regimes. Concentration of 100 mM NaCl turned out to be toxic, with up to 89.1% shoot fresh weight losses, in comparison to unchallenged with salt stress seedlings (Table 1, Figure 1).

Table 1. Number of lateral shoots formed, shoot and root fresh weights (g), shoot and root dry weights (g) and root/shoot ratio in *P. spinosa* seedlings upon saline soil stress.

	Saline Soil Stress (NaCl)	Lateral Shoot Number	Shoot Fresh Weight (g)	Root Fresh Weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)	Root/Shoot Ratio
No stimulatory Supplementation	Non-stressed	$8.20 \pm 1.49^{a,a}$	$12.67 \pm 1.70^{a,a}$	$8.86 \pm 1.42^{a,a}$	$6.73 \pm 1.26^{a,a}$	$3.81 \pm 0.72^{a,a}$	$0.56 \pm 0.02^{a,a}$
	Salt stress 50 mM	$5.20 \pm 1.53^{a,b}$	$6.92 \pm 0.63^{a,b}$	$7.42 \pm 0.87^{a,a}$	$3.33 \pm 0.36^{a,b}$	$2.78 \pm 0.26^{a,a}$	$0.84 \pm 0.06^{a,b}$
	Salt stress 75 mM	$1.60 \pm 0.87^{a,c}$	$2.46 \pm 0.59^{a,c}$	$3.58 \pm 0.66^{a,b}$	$1.42 \pm 0.29^{a,c}$	$1.16 \pm 0.21^{a,b}$	$0.93 \pm 0.24^{a,b}$
	Salt stress 100 mM	$0.00 \pm 0.00^{a,d}$	$1.48 \pm 0.21^{a,d}$	$2.94 \pm 0.21^{a,b}$	$1.14 \pm 0.16^{a,c}$	$0.94 \pm 0.06^{a,b}$	$0.88 \pm 0{,}12^{a,b}$
Amino acid Supplementation	Non-stressed	$4.40 \pm 0.40^{b,a}$	$10.78 \pm 1.12^{b,a}$	$8.77 \pm 0.91^{a,a}$	$4.72 \pm 0.52^{a,a}$	$3.62 \pm 0.41^{a,a}$	$0.77 \pm 0.07^{b,a}$
	Salt stress 50 mM	$1.80 \pm 0.58^{b,b}$	$4.80 \pm 0.76^{b,b}$	$6.88 \pm 0.68^{a,a}$	$2.31 \pm 0.38^{b,b}$	$2.40 \pm 0.23^{ab,b}$	$1.09 \pm 0.10^{b,b}$
	Salt stress 75 mM	$0.00 \pm 0.00^{b,c}$	$2.73 \pm 0.66^{a,c}$	$4.47 \pm 0.36^{a,b}$	$1.13 \pm 0.14^{a,c}$	$1.55 \pm 0.12^{b,c}$	$1.42 \pm 0.16^{b,c}$
	Salt stress 100 mM	$0.60 \pm 0.40^{a,d}$	$2.34 \pm 0.58^{b,c}$	$3.56 \pm 0.55^{a,b}$	$1.43 \pm 0.37^{a,c}$	$1.23 \pm 0.22^{a,c}$	$1.16 \pm 0.38^{a,b}$
Microbial Supplementation	Non-stressed	$4.00 \pm 0.83^{b,a}$	$7.47 \pm 1.33^{c,a}$	$6.71 \pm 1.01^{a,a}$	$3.18 \pm 0.58^{b,a}$	$2.51 \pm 0.38^{b,a}$	$0.83 \pm 0.08^{b,a}$
	Salt stress 50 mM	$1.40 \pm 0.97^{b,b}$	$5.74 \pm 1.34^{a,a}$	$8.84 \pm 0.66^{a,b}$	$2.69 \pm 0.60^{b,a}$	$3.16 \pm 0.26^{ac,ab}$	$1.34 \pm 0.19^{c,b}$
	Salt stress 75 mM	$0.00 \pm 0.00^{b,c}$	$2.51 \pm 0.46^{a,b}$	$5.00 \pm 0.85^{a,a}$	$1.43 \pm 0.16^{a,b}$	$1.78 \pm 0.33^{b,ac}$	$1.23 \pm 0.16^{b,b}$
	Salt stress 100 mM	$1.80 \pm 0.66^{b,b}$	$1.39 \pm 0.22^{a,c}$	$3.10 \pm 0.73^{a,c}$	$1.04 \pm 0.14^{a,b}$	$1.10 \pm 0.19^{a,d}$	$1.11 \pm 0.27^{a,b}$

The different letters indicate a significant ($p < 0.05$) difference; first letter demonstrates differences between exogenous treatments and the second one among salt stress levels. Data are presented as the mean ± SE of five replicates.



Figure 1. *P. spinosa* seedlings: (**A**) Non-salt-treated plantlets, and (**B**–**D**) plantlets treated with 50 mM, 75 mM and 100 mM NaCl soil stress, respectively. From each section in left to right direction, non-stimulatory supplementation, amino acid- and microbial-supplemented plantlet lines.

Root fresh weights for the same levels of salinity stress were not affected by the type of exogenous alleviation treatment. Salt stress level of 50 mM NaCl independently of soil treatment conferred root fresh weight values close to non-stressed plantlets; values exhibited sharp decline above 50% upon 75 mM NaCl exposure, and they reached an absolute weakness plateau on the highest applied concentration (100 mM NaCl) (Table 1).

3.2. Shoot and Root Dry Weights

Higher shoot dry weight values were recorded in non-supplemented and l-methionine soil-treated plantlets. Bacterial application in zero salt presence condition provided the lowest shoot dry weight. A total of 50 mM NaCl salt stress provoked reduction in shoot dry weight at both exogenous applications more than in untreated plantlets. Salt stress of 75 mM and 100 mM NaCl provided—in a horizontal pattern—minimum shoot dry weight values for all treatments without outcome differences to play a significant role. Total decline of shoot dry weight in presence of 100 mM NaCl salt stress reached maximum of 83.1%, 69.70% and 67.29% for non-treated, amino acid-treated and bacterial-treated plantlets in respect to unchallenged salt status (Table 1).

At non-stress-challenged saline status, l-methionine soil treatment did not provide any change in root dry weight of plantlets while microbial application affected it in a negative manner. Independently of the presence (or not) of exogenous saline stress alleviation scheme, the highest applied salt concentration (100 mM NaCl) resulted in a sharp drop in root dry weight (24.67–43.82%). Nevertheless, microbial salt stress alleviation at 50 mM NaCl performed as well as non-treated and l-methionine-treated plantlets, moving towards lower root dry weight losses due to stress. Salt stress levels of 75 mM and 100 mM NaCl induced similar dry root biomass development in non-supplemented and amino acid-treated plantlets, with minor significant differences to be apparent in microbial stress alleviation scheme (Table 1).

3.3. Root/Shoot Ratio

Both exogenous applications increased root/shoot ratio above 1.00 in salt stress conditions in all salt concentrations providing developmental boost for root growth. In the highest salt concentration applied, root growth boosted over shoot growth at 50.64% and 33.73% for l-methionine biostimulant and bacterial supplementation, respectively. The lowest comparative shoot growth was observed in 75 mM NaCl soil conditions for unchallenged, non-supplemented and amino acid-treated plantlets. Microbial-treated plantlets exhibited the same root/shoot ratio pattern effect for graded salt stress levels from 50 mM to 100 mM NaCl (Table 1).

3.4. Plant Height

Concerning stress level effect on unchallenged saline plantlets, those treated with l-methionine and bacterial applications appeared to limit plant height attribute during the tested months (April–July). Plant height on salt unchallenged conditions appeared to be lower in amino acid- and microbial-supplemented lines by 9.17–25.04% and 29.13–37.65% in relation to untreated ones for the same time period. This trend of low plant height performance on exogenously treated seedlings was also observed at 50 mM NaCl (13.18–19.38% for l-methionine and 13.18–25.26% bacterial supplementation losses, respectively). From April to May, *P. spinosa* plantlets under 75 mM NaCl soil conditions, which were treated with amino acid and bacterial means, had overcome in height the non-supplemented ones. Thereafter (i.e., June–July), equal plant height patterns were followed by all plantlets independently of their treatment. Salt stress at 100 mM NaCl exhibited, among all treatments and time intervals (months), similar low plant height values reflecting high levels of growth stress (Table 2).

In terms of salt exposure time effect, in April, all plantlets independent of treatment type demonstrated a declined growth rate at the highest soil saline concentration (100 mM NaCl) and better plant height growth observed on l-methionine-treated plantlets; whereas, microbial and non-treated plantlets did not differ significantly. However, for the same month, 50 mM NaCl saline inhibitory effect on plant height was not alleviated by exogenous treatments; whereas, 75 mM NaCl conferred resilience in comparison to untreated seedlings. In May, under 50 mM and 75 mM NaCl soil stress conditions, amino acid- and microbial-treated plantlets performed better than untreated ones. Apart from unchallenged plantlets, June and July were two months where *P. spinosa* exhibited low growth values due to applicable soil stress. From 0 to 100 mM NaCl soil salt stress for the month of July, plant

height growth inhibited at the levels of 52.75%, 45.79% and 41.58% for non-supplemented, amino acid-treated and microbial-treated plantlets, respectively (Table 2).

Table 2. Plant height (cm) of *P. spinosa* seedlings upon saline soil stress.

Saline Soil Stress (NaCl)	Month	No Stimulatory Supplementation	Supplementation	
			Amino Acid	Microbial
Non-stressed	April	13.18 ± 0.72 ^{a,a,a}	10.30 ± 0.52 ^{b,a,a}	8.40 ± 0.34 ^{c,a,a}
	May	22.44 ± 1.78 ^{a,b,a}	16.82 ± 0.93 ^{b,b,a}	15.06 ± 0.69 ^{c,b,a}
	June	35.00 ± 2.63 ^{a,c,a}	30.94 ± 1.82 ^{a,c,a}	21.82 ± 1.93 ^{b,c,a}
	July	41.40 ± 3.14 ^{a,d,a}	37.60 ± 3.20 ^{b,d,a}	29.34 ± 2.55 ^{c,d,a}
Salt stress 50 mM	April	13.80 ± 1.05 ^{a,a,a}	11.56 ± 0.61 ^{b,a,a}	11.72 ± 0.99 ^{b,a,b}
	May	22.68 ± 1.83 ^{a,b,a}	19.48 ± 1.46 ^{b,b,b}	21.14 ± 1.54 ^{ab,b,b}
	June	28.98 ± 1.72 ^{a,c,b}	25.16 ± 1.44 ^{b,c,b}	25.16 ± 1.66 ^{b,c,a}
	July	34.04 ± 3.22 ^{a,d,b}	27.44 ± 1.46 ^{b,d,b}	25.44 ± 1.59 ^{b,c,b}
Salt stress 75 mM	April	8.82 ± 0.70 ^{a,a,b}	11.02 ± 0.21 ^{b,a,a}	11.30 ± 0.75 ^{b,a,b}
	May	15.66 ± 1.42 ^{a,b,b}	18.82 ± 0.66 ^{b,b,b}	18.00 ± 0.46 ^{b,b,c}
	June	21.48 ± 2.39 ^{a,c,c}	22.26 ± 2.42 ^{a,c,bc}	22.14 ± 1.76 ^{a,c,a}
	July	21.48 ± 2.39 ^{a,c,c}	22.68 ± 2.74 ^{a,c,c}	22.16 ± 1.75 ^{a,c,b}
Salt stress 100 mM	April	8.68 ± 0.27 ^{a,a,b}	10.94 ± 0.48 ^{b,a,a}	8.54 ± 0.36 ^{a,a,b}
	May	17.88 ± 0.75 ^{a,b,b}	17.34 ± 1.13 ^{a,b,b}	15.80 ± 0.83 ^{a,b,a}
	June	19.56 ± 0.56 ^{a,c,c}	19.98 ± 1.91 ^{a,b,c}	17.14 ± 1.63 ^{a,b,b}
	July	19.56 ± 0.56 ^{a,c,c}	20.38 ± 1.84 ^{a,b,c}	17.14 ± 1.63 ^{a,b,c}

The different letters indicate a significant ($p < 0.05$) difference; first letter demonstrates differences between exogenous treatments for the same monthly period and the same level of saline stress, second demonstrates differences between different monthly periods for the same levels of saline stress and exogenous treatment and third letter demonstrates statistical differences for the same monthly period among different levels of salt stress for the same exogenous treatment.

3.5. Total Phenolic Content

No differences were recorded in leaf phenolic content among treatments and levels of saline stress. All mean values were found to be between 14.02 (non-treated plantlet at stress levels of 75 mM NaCl) and 14.81 (microbial-treated plantlets at stress levels of 50 mM NaCl) mg GAE/g d.w., resulting in no differences between salt stress levels and modes of potential salinity alleviation treatments (Figure 2).

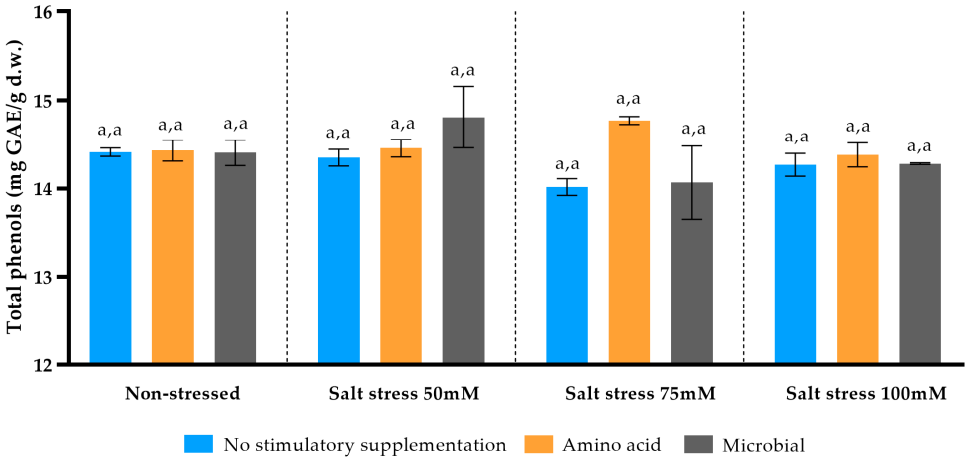

Figure 2. Total phenolic content (mg GAE/g d.w.) in *P. spinosa* seedlings upon saline soil stress. The different letters indicate a significant ($p < 0.05$) difference; first letter demonstrates differences between exogenous treatments and the second one among salt stress levels. Data are presented as the mean ± SE of five replicates.

3.6. Total Chlorophyll Content

Total chlorophyll content values (May–July) did not differ among unchallenged saline plantlets independently of exogenous treatment presence for the same time period. A decline to a plateau level was observed in June and July. Chlorophyll presence was poorer and decline was greater at 50 mM NaCl stress, reaching a narrow value plateau in the last two consecutive months (mean value drop for June–July: 66.8–72.1%) with minor restorative statistical exception in amino acid supplementation. Plateau was also reached at 75 mM and 100 mM NaCl soil salinity stress for all plantlets, achieving new low for chlorophyll values (63.57–78.04% drop for all experimental plantlet lines, independently of exogenous treatment type and presence). Data revealed a non-specific effect (50–100 mM NaCl) on chlorophyll activity no matter what salinity alleviation scheme was applied (Table 3).

Table 3. Total chlorophyll content (SPAD values) of *P. spinosa* leaves upon saline soil stress.

Saline Soil Stress (NaCl)	Month	No Stimulatory Supplementation	Supplementation	
			Amino Acid	Microbial
Non-stressed	May	$7.64 \pm 0.42^{a,a,a}$	$7.16 \pm 1.12^{a,a,a}$	$4.60 \pm 1.03^{b,a,a}$
	June	$4.54 \pm 0.70^{a,b,a}$	$3.34 \pm 0.47^{a,b,a}$	$3.86 \pm 0.60^{a,a,a}$
	July	$4.24 \pm 0.43^{a,b,a}$	$3.64 \pm 0.40^{a,b,a}$	$4.94 \pm 0.60^{a,a,a}$
Salt stress 50 mM	May	$5.42 \pm 0.69^{a,a,b}$	$4.58 \pm 0.62^{b,a,b}$	$4.10 \pm 0.55^{b,a,a}$
	June	$1.74 \pm 0.21^{a,b,b}$	$1.40 \pm 0.35^{a,b,b}$	$1.38 \pm 0.14^{a,b,b}$
	July	$1.56 \pm 0.17^{a,b,b}$	$1.50 \pm 0.11^{a,c,b}$	$1.36 \pm 0.10^{a,b,b}$
Salt stress 75 mM	May	$3.28 \pm 0.33^{a,a,c}$	$3.56 \pm 0.68^{a,a,b}$	$2.80 \pm 0.50^{a,a,b}$
	June	$1.50 \pm 0.07^{a,b,b}$	$1.30 \pm 0.19^{ab,b,b}$	$1.30 \pm 0.10^{b,b,b}$
	July	$0.72 \pm 0.10^{a,c,c}$	$1.14 \pm 0.05^{b,c,c}$	$1.02 \pm 0.09^{b,b,b}$
Salt stress 100 mM	May	$2.50 \pm 0.57^{a,a,c}$	$2.40 \pm 0.32^{a,a,c}$	$1.88 \pm 0.11^{b,a,c}$
	June	$1.08 \pm 0.07^{a,b,c}$	$0.98 \pm 0.08^{a,b,c}$	$1.00 \pm 0.12^{a,b,c}$
	July	$1.06 \pm 0.02^{a,b,d}$	$0.96 \pm 0.08^{a,b,d}$	$0.68 \pm 0.08^{b,c,c}$

The different letters indicate a significant ($p < 0.05$) difference; first letter demonstrates differences between exogenous treatments for the same monthly period and the same level of saline stress, second demonstrates differences between different monthly periods for the same levels of saline stress and exogenous treatment and third letter demonstrates statistical differences for the same monthly period among different levels of salt stress for the same exogenous treatment. Data are presented as the mean ± SE of five replicates.

3.7. Leaf Antioxidant Activity

All experimental plantlet lines increased their antioxidant activity in presence of soil NaCl enrichment. Non-supplemented plantlets exhibited greater range of antioxidant activity values in comparison to those that were exogenously l-methionine- or bacteria-soil-enriched. Up to 100 mM NaCl salt stress, plantlets increased their antioxidant activity 3.6, 1.4 and 1.6 times at non-supplemented, amino acid- and bacterial-supplemented lines, respectively. The lowest antioxidant response was recorded for 50 mM NaCl amino acid-treated seedlings, which did not seem to differ from unchallenged status. The antioxidant response to upscaled saline stress (75 mM, 100 mM NaCl) appeared to be non-specific among supplemented and non-supplemented plantlet lines (Figure 3).

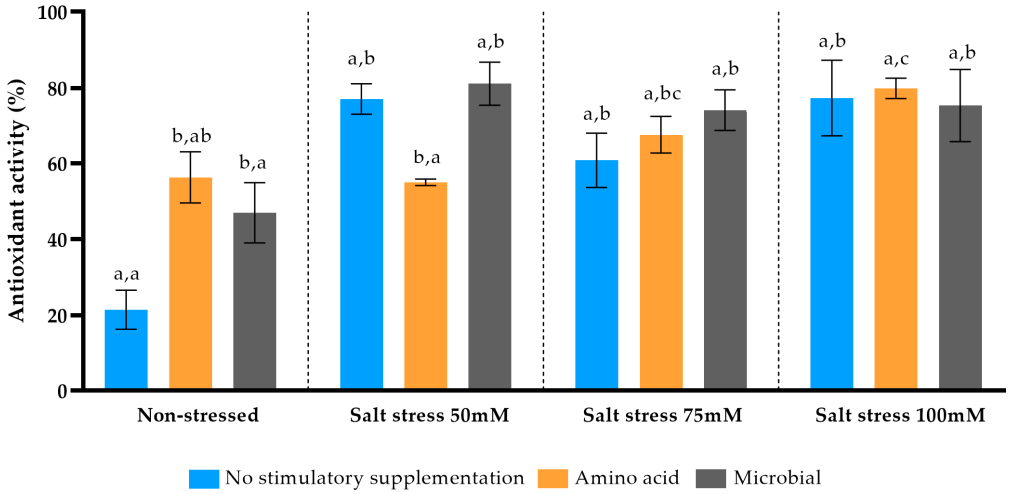

Figure 3. Antioxidant activity (%) found on *P. spinosa* leaves upon saline soil stress. The different letters indicate a significant ($p < 0.05$) difference; first letter demonstrates differences between exogenous treatments and the second one among salt stress levels. Data are presented as the mean ± SE of five replicates.

3.8. Quantification of Changes in Root Size and Architecture

For the same levels of salinity stress, the median number of roots did not differ. Non-supplemented plantlets increased their median number of roots while saline stress scaled up. This trend was not apparent for amino acid- and bacterial-treated seedlings where no differences were recorded (Table 4, Figures 4–6).

Table 4. Median root number, root tip number, total root length (mm), projected root volume (cm^3) and root orientation (degrees) found on *P. spinosa* seedlings upon saline soil stress.

	Saline Soil Stress (NaCl)	Median Root Number	Root Tip Number	Total Root Length (mm)	Projected Root Volume (cm^3)	Root Orientation (Degrees)
No stimulatory Supplementation	Non-stressed	14.40 ± 2.25 ^{a,a}	3444 ± 623 ^{a,a}	10,452 ± 1728 ^{a,a}	51.90 ± 11.29 ^{a,a}	45.05 ± 0.74 ^{a,a}
	Salt stress 50 mM	16.20 ± 2.13 ^{a,ab}	3831 ± 506 ^{a,a}	11,213 ±1482 ^{a,a}	115.38 ± 46.83 ^{a,a}	45.21 ± 0.57 ^{a,a}
	Salt stress 75 mM	16.40 ± 2.83 ^{a,ab}	3489 ± 571 ^{a,a}	8858 ± 1260 ^{a,a}	19.98 ± 4.42 ^{a,b}	46.40 ± 1.04 ^{a,a}
	Salt stress 100 mM	21.20 ± 3.29 ^{a,b}	3791 ± 480 ^{a,a}	9457 ± 990 ^{a,a}	18.72 ± 6.05 ^{a,b}	44.96 ± 0.65 ^{a,a}
Amino acid Supplementation	Non-stressed	18.80 ± 2.71 ^{a,a}	5489 ± 1670 ^{a,a}	16,586 ± 3239 ^{b,a}	109.99 ± 30.50 ^{b,a}	44.82 ± 0.35 ^{a,a}
	Salt stress 50 mM	16.80 ± 2.17 ^{a,a}	4989 ± 487 ^{b,a}	12,974 ± 1387 ^{a,a}	62.11 ± 22.05 ^{ab,a}	43.85 ± 0.61 ^{a,a}
	Salt stress 75 mM	17.40 ± 2.04 ^{a,a}	4120 ± 453 ^{a,a}	9878 ± 646.5 ^{a,b}	44.59 ± 10.50 ^{b,b}	43.76 ± 0.68 ^{a,a}
	Salt stress 100 mM	18.20 ± 3.27 ^{a,a}	4210 ± 433 ^{a,a}	9493 ± 618.5 ^{a,b}	37.79 ± 22.33 ^{a,b}	46.20 ± 0.75 ^{a,a}
Microbial Supplementation	Non-stressed	13.66 ± 4.31 ^{a,a}	4824 ± 788 ^{a,a}	12,494 ± 2162 ^{ab,ab}	101.55 ± 29.92 ^{b,a}	46.14 ± 0.98 ^{a,a}
	Salt stress 50 mM	17.72 ± 5.25 ^{a,a}	6588 ± 1005 ^{c,b}	16,178 ± 1751 ^{b,a}	48.45 ± 7.49 ^{b,b}	44.46 ± 0.45 ^{a,a}
	Salt stress 75 mM	15.60 ± 2.76 ^{a,a}	4590 ± 562 ^{a,a}	10,919 ± 1033 ^{a,b}	24.39 ± 4.32 ^{ac,c}	46.00 ± 0.68 ^{a,a}
	Salt stress 100 mM	14.10 ± 1.79 ^{a,a}	2832 ± 355 ^{b,c}	7167 ± 1079 ^{b,c}	32.38 ± 16.95 ^{a,bc}	43.82 ± 0.71 ^{a,a}

The different letters indicate a significant ($p < 0.05$) difference; first letter demonstrates differences between exogenous treatments and the second one among salt stress levels. Data are presented as the mean ± SE of five replicates.

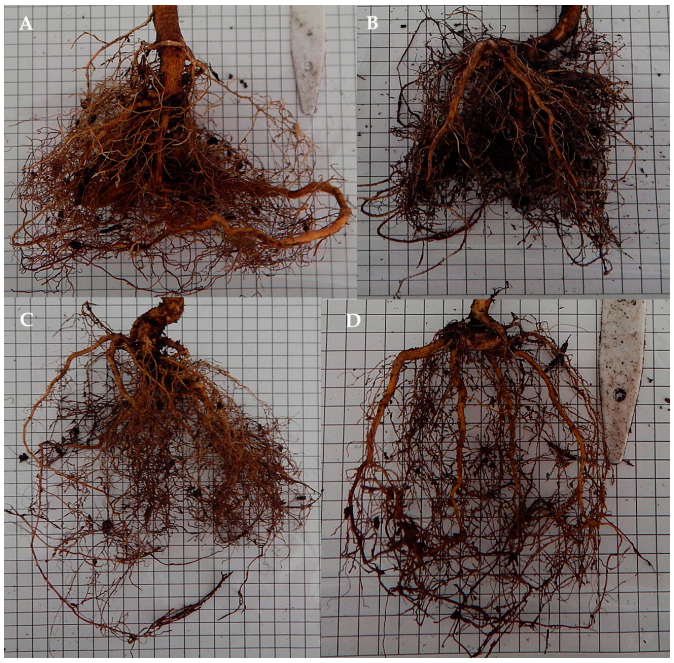

Figure 4. Roots of *P. spinosa* seedlings without amino acid or microbial supplementation: (**A**) Non-salt-treated plantlets, and (**B**–**D**) plantlets treated with 50 mM, 75 mM and 100 mM NaCl soil stress, respectively.



Figure 5. Roots of *P. spinosa* seedlings with amino acid supplementation: (**A**) Non-salt-treated plantlets, and (**B**–**D**) plantlets treated with 50 mM, 75 mM and 100 mM NaCl soil stress, respectively.



Figure 6. Roots of *P. spinosa* seedlings with microbial supplementation: (**A**) Non-salt-treated plantlets, and (**B**–**D**) plantlets treated with 50 mM, 75 mM and 100 mM NaCl soil stress, respectively.

The number of root tips was not suppressed for all stressed plantlet lines. Microbial support in *P. spinosa* plantlets induced increased formation of root tips among other treatments on lowest applied salinity stress level (50 mM NaCl); however, this attribute was not followed in more severe saline conditions (75 mM, 100 mM NaCl) (Table 4).

Increased salinity stress levels in *P. spinosa* seedlings reduced total root length in severe salt stress conditions among supplemented lines. Amino acid and microbial treatments provided high value picks in total root length at saline unchallenged condition and 50 mM NaCl-challenged soil conditions. These experimental lines under 75 mM and 100 mM NaCl saline stress provided a statistically significant decline in comparison to initially unchallenged status (Table 4).

Root average diameter in all experimental lines declined as soil salt stress level increased (75 mM and 100 mM NaCl). Average root diameter for non-supplemented, l-methionine- and bacteria-supplemented plantlets dropped from unchallenged status to 100 mM NaCl salt stress by 38.65%, 28.93% and 29.21%, respectively. Root median diameter reduction in scaled up saline stress, independently of supplementation, emerged as a unified response to the stress factor. Values for non-supplemented, amino acid- and microbial-supplemented plantlets dropped from unchallenged status to 100 mM NaCl soil stress condition by 35.86%, 26.5% and 24.17%, respectively. Root maximum diameter data patterns were differentiated in presence of exogenous treatments and *P. spinosa* survival strategy per level of scaled stress. Non-supplemented plantlets exhibited high maximum diameter values at 50 mM NaCl salt stress, which declined sharply thereafter at 75 mM and 100 mM NaCl soil exposure. Amino acid-treated plants followed also the same decline trend up to 100 mM NaCl salt stress. Microbial-treated plants declined their maximum diameter roots, reaching a value plateau from 50 mM NaCl concentration (Table 5).

Lower projected root volumes were recorded in saline environments for all plantlet experimentation lines especially at equal or higher concentrations of 75 mM NaCl exposure. Non-supplemented plantlets exhibited a similar projected root volume at 100 mM NaCl as compared to amino acid- and microbial-treated ones. Amino acid- and microbial-treated *P. spinosa* seedlings under zero saline stress soil conditions increased more than

50% their root volume; however, this type of shift was recorded for unchallenged to salt, non-supplemented seedlings, when exposed to 50 mM NaCl stress. The 100 mM NaCl salt stress condition resulted in 50.46% and 42.18% greater root projected volumes for amino acid- and microbial-treated seedlings, respectively, in comparison to non-treated ones (Table 4, Figures 4–6).

Table 5. Maximum (MX), average (AV) and median (MD) root diameters (mm) found on *P. spinosa* seedlings upon saline soil stress.

Saline Soil Stress (NaCl)	Root Diameter	No Stimulatory Supplementation	Supplementation	
			Amino Acid	Microbial
Non-stressed	MX	15.73 ± 1.69 ^{a,a}	21.15 ± 1.85 ^{b,a}	22.79 ± 3.74 ^{b,a}
Salt stress 50 mM		23.09 ± 4.88 ^{a,b}	16.35 ± 3.21 ^{ab,a}	14.59 ± 1.33 ^{b,b}
Salt stress 75 mM		11.26 ± 2.29 ^{a,c}	16.86 ± 3.04 ^{b,a}	12.17 ± 1.36 ^{a,b}
Salt stress 100 mM		9.71 ± 1.55 ^{a,c}	13.82 ± 4.42 ^{a,b}	13.38 ± 3.74 ^{a,b}
Non-stressed	AV	1.63 ± 0.25 ^{a,a}	1.59 ± 0.07 ^{a,a}	1.78 ± 0.25 ^{a,a}
Salt stress 50 mM		1.98 ± 0.48 ^{a,a}	1.43 ± 0.20 ^{ab,ab}	1.23 ± 0.11 ^{b,b}
Salt stress 75 mM		1.05 ± 0.07 ^{a,b}	1.31 ± 0.12 ^{b,b}	1.04 ± 0.07 ^{a,b}
Salt stress 100 mM		1.00 ± 0.12 ^{a,b}	1.13 ± 0.25 ^{a,b}	1.26 ± 0.16 ^{a,b}
Non-stressed	ME	0.92 ± 0.13 ^{a,a}	0.83 ± 0.08 ^{a,a}	0.91 ± 0.10 ^{a,a}
Salt stress 50 mM		0.84 ± 0.05 ^{a,a}	0.78 ± 0.08 ^{ab,ab}	0.74 ± 0.04 ^{b,b}
Salt stress 75 mM		0.63 ± 0,02 ^{a,b}	0.71 ± 0.04 ^{a,b}	0.63 ± 0.02 ^{a,c}
Salt stress 100 mM		0.59 ± 0.04 ^{a,b}	0.61 ± 0.06 ^{a,b}	0.69 ± 0.05 ^{a,b}

The different letters indicate a significant ($p < 0.05$) difference; first letter demonstrates differences between exogenous treatments and the second one among salt stress levels. Data are presented as the mean ± SE of five replicates.

Root orientation via average angle metrics did not differ based on exogenous treatments and levels of soil salt stress (high/low values between 43.11° and 47.44°) (Table 4). Frequencies for root angles below 30 degrees did not differ (average frequency 33.67%) among non-supplemented and amino acid-supplemented plantlets in all soil salt stress levels. Also, frequencies of root angles in grouped sets of 30°–60° and 60°–90° did not differ independently of treatment and levels of soil salt stress (average frequency 31.42% and 34.88%, respectively) (Table 6).

Table 6. Root angle frequencies (%) found on *P. spinosa* seedlings upon saline soil stress.

Saline Soil Stress (NaCl)	Angle	No Stimulatory Supplementation	Supplementation	
			Amino Acid	Microbial
Non-stressed	≤30 degrees	32.60 ± 1.28 ^{a,a}	34.40 ± 0.81 ^{a,a}	31,20 ± 1.24 ^{a,a}
Salt stress 50 mM		33.00 ± 0.89 ^{a,a}	35.00 ± 1.14 ^{a,ab}	34.40 ± 0.87 ^{a,ab}
Salt stress 75 mM		32.60 ± 1.20 ^{a,a}	36.00 ± 0.89 ^{b,b}	33.20± 0.91 ^{a,a}
Salt stress 100 mM		35,00 ± 0.63 ^{a,b}	33.00 ± 1.04 ^{a,a}	36.40 ± 1.20 ^{a,b}
Non-stressed	≤60 degrees	33.40 ± 1.96 ^{a,a}	31.80 ± 0.49 ^{a,a}	33.40 ± 2.04 ^{a,a}
Salt stress 50 mM		33.00 ± 0.63 ^{a,a}	32.80 ± 1.28 ^{a,a}	32.20 ± 1.24 ^{a,a}
Salt stress 75 mM		29.60 ± 0.74 ^{a,b}	30.60 ± 0.50 ^{a,b}	30.20 ± 0.37 ^{a,b}
Salt stress 100 mM		29.40 ± 0.74 ^{a,b}	29.80 ± 1.06 ^{a,b}	30.00 ± 0.83 ^{a,b}
Non-stressed	≤90 degrees	33.80 ± 1.56 ^{a,a}	34.00 ±0.44 ^{a,a}	35.60 ± 2.11 ^{a,a}
Salt stress 50 mM		34.20 ± 1.02 ^{a,a}	32.60 ± 1.12 ^{a,a}	33.60 ± 1.12 ^{a,ab}
Salt stress 75 mM		37.60 ± 1.6 ^{a,b}	33.40 ± 1.07 ^{b,a}	37.00 ± 1.04 ^{a,ac}
Salt stress 100 mM		35.80 ± 1.24 ^{a,ab}	37.40 ± 1.47 ^{a,b}	33.60 ± 1.03 ^{a,ab}

The different letters indicate a significant ($p < 0.05$) difference; first letter demonstrates differences between exogenous treatments and the second one among salt stress levels. Data are presented as the mean ± SE of five replicates.

At non-saline-stressed conditions, total root lengths, independently of their thicknesses, were longer in amino acid soil supplementation followed by microbial, while the control's plantlets had lower performance. Roots with smaller thickness size produce larger-in-length root networks in comparison to thick ones. Based on root thickness size classification (1–≥6 mm), total root length values dropped in presence of saline stress for all plantlet lines, minimizing the differences as the levels of salt stress increased (Table 7).

Table 7. Total length of roots (mm) per root thickness diameter found on *P. spinosa* seedlings upon saline soil stress.

Saline Soil Stress (NaCl)	Root Thickness (mm)	No Stimulatory Supplementation	Supplementation	
			Amino Acid	Microbial
Non-stressed	1	5836 ± 1391 ^{a,a,a}	9237 ± 1988 ^{b,a,a}	6641 ± 1462 ^{a,a,a}
	2	2207 ± 389 ^{a,b,a}	3468 ± 653 ^{b,b,a}	2823 ± 636 ^{a,b,ab}
	3	937.7 ± 136.8 ^{a,c,a}	1473 ± 245.4 ^{b,c,a}	1122 ± 186.2 ^{b,c,a}
	4	537.2 ± 58.46 ^{a,d,a}	818.3 ± 149.5 ^{b,d,a}	606.7 ± 110.2 ^{a,d,a}
	5	296.9 ± 51.66 ^{a,e,a}	482.8 ± 90.52 ^{b,e,a}	370.3 ± 72.72 ^{a,e,a}
	≥6	636.6 ± 191.3 ^{a,d,a}	1107 ± 259.5 ^{b,cd,a}	931.3 ± 287.3 ^{b,d,a}
Salt stress 50 mM	1	6139 ± 911 ^{a,a,a}	7822 ± 1547 ^{ab,a,ab}	9981 ± 1433 ^{b,a,b}
	2	2401 ± 427 ^{a,b,a}	2635 ± 141 ^{b,b,b}	3606 ± 427 ^{c,b,a}
	3	972.3 ± 180.9 ^{a,c,a}	1012 ± 67.18 ^{ab,c,b}	1228 ± 78.68 ^{b,c,a}
	4	515.6 ± 87.43 ^{a,d,a}	542.6 ± 45.60 ^{a,d,b}	565.0 ± 41.45 ^{a,d,a}
	5	315.4 ± 45.12 ^{a,e,a}	321.0 ± 35.70 ^{a,e,b}	285.2 ± 32.71 ^{a,e,a}
	≥6	870.0 ± 167.2 ^{a,c,a}	641.2 ± 183.2 ^{ab,d,b}	513.1 ± 111.4 ^{b,d,b}
Salt stress 75 mM	1	6003 ± 800 ^{a,a,a}	6203 ± 613.3 ^{a,a,b}	7435 ± 696.9 ^{a,a,a}
	2	1760 ± 316.5 ^{a,b,a}	1971 ± 105.4 ^{a,b,c}	2107 ± 253.2 ^{a,b,b}
	3	532.4 ± 108.5 ^{a,c,b}	645.4 ± 66.60 ^{a,c,c}	659.1 ± 75.51 ^{a,c,b}
	4	256.8 ± 54.27 ^{a,d,b}	355.9 ± 45.67 ^{a,d,c}	312.3 ± 37.53 ^{a,d,b}
	5	129.2 ± 19.89 ^{a,e,b}	215.2 ± 34.08 ^{b,e,c}	159.8 ± 23.18 ^{a,e,b}
	≥6	72.24 ± 9.59 ^{a,f,b}	488.6 ±108.6 ^{b,c,b}	245.5 ± 67.71 ^{c,de,c}
Salt stress 100 mM	1	6645 ± 856 ^{a,a,a}	6663 ± 801 ^{a,a,b}	4408 ± 672 ^{b,a,c}
	2	1704 ± 226.2 ^{a,b,a}	1689 ± 69.95 ^{a,b,d}	1439 ± 206.4 ^{a,b,c}
	3	532.1 ± 72.67 ^{a,c,b}	511.2 ± 41.28 ^{a,c,d}	569.2 ± 110.2 ^{a,c,b}
	4	254.8 ± 55.90 ^{a,d,b}	233.4 ± 26.68 ^{a,d,d}	289.1 ± 54.09 ^{a,d,b}
	5	135.9 ± 37.11 ^{a,e,b}	121.6 ± 22.07 ^{a,e,d}	167.0 ± 32.18 ^{a,e,b}
	≥6	185.0 ± 88.96 ^{a,e,c}	275.3 ± 137.8 ^{a,d,c}	294.3 ± 132.2 ^{a,d,c}

The different letters indicate a significant ($p < 0.05$) difference; first letter demonstrates differences between exogenous treatments for the same root thickness size and the same level of saline stress, second demonstrates differences between different root thickness sizes for the same levels of saline stress and exogenous treatment and third letter demonstrates statistical differences for the same root thickness size among different levels of salt stress for the same exogenous treatment. Data are presented as the mean ± SE of five replicates.

4. Discussion

Sodium chloride-based salinity affected negatively the number of shoots in many tree species. Six-month-old pomegranate plantlets dropped their lateral shoots number by 11.36%, 50% and 61.36% for 40 mM, 80 mM and 120 mM NaCl stress, respectively; herein, *P. spinosa* declined faster under the same type of salt stress challenge [41]. The osmotic effect of NaCl when salt is accumulated in soil can explain these biomass losses [42]. Soil exogenous applications of l-methionine and microorganisms did not favor lateral shoot formation even at non-saline challenge condition. Methionine needs sulfur-rich proteins for long-distance transport and loading into phloem with complex adjustments in sulfur availability at organismal level; leaves are more competent organs for these tasks when induced systemic reactions take place [43,44]. In addition, when phloem contains elevated levels of methionine, it triggers an increased presence of sucrose, which is a major counter-saline osmolyte [44]. Herein, soil application of methionine was possibly restricted towards nutritional absorption and stimulation of endogenous hormone homeostasis in

roots; functional weakness of methionine sulfoxide reductase mechanism to reinstate methionine from its oxidative state under saline stress conditions could also be taken under consideration for low alleviation performance of the molecule [19,25].

In contrast, foliar application of methionine was found to (a) alleviate drought and saline osmotic-related stress in other plant species, enhancing shoot growth [22,45], and (b) induce l-methionine plant signaling to enhance salt tolerance, jointly with soil presence of *Bacillus subtilis* strains [46].

Shoot fresh weights declined upon saline presence, which is in accordance with similar stress experimentation in apple rootstock MM.106 [47]. Herein, soil application of methionine alleviated the negative effect on shoot weight as it is shown in foliar application in non-horticultural species (e.g., maize), suggesting a universal protecting role for this molecule no matter which way it will be exogenously administrated on the plant host [48]. Bacterial counteraction to NaCl stress on shoot fresh weight was not successful as compared to in vitro apple tree saline challenge [49]. *P. spinosa* inhibitory effect on shoot biomass at 75 mM and especially at 100 mM NaCl levels of soil stress were also demonstrated in at least three varieties of olive trees [50].

Neither soil l-methionine nor microbial application were capable to ameliorate root fresh weight values. However, in proportionally less-lignified plant species (e.g., maize) than *P. spinosa*, l-methionine tends to reverse ionic imbalance and restore roots fresh weight standards when administered foliarly in saline environments [48]. Similarly, microbial soil support of *Bacillus* species increases root weight characteristics under salt stress [51,52]. As seen above, there are many similar patterns of *P. spinosa* seedling fresh weight changes with other plant species under salt stress; however, some researchers propose comparative data avoidance due to several abiotic and biotic uncontrolled conditions when data were collected [53].

Lower performance in shoot dry weight formation of unchallenged plantlets in presence of l-methionine may be linked to cell membrane and protein synthesis functional disruption due to their overwhelming presence [54]. Dry weight minimal losses under salinity stress have also been documented well in field crops where previous data are available [55–57].

Root dry weight did not reveal changes among exogenous treatments in the presence of increased salt stress; however, percentage (%) losses in root weight were fewer than those in shoots. This is also in accordance with root/shoot ratio data examination, emerging a potential tissue plasticity survival mechanism. Above a value of 1.00, the root/shoot ratio of *P. spinosa* seedlings in enriched bacterial and l-methionine saline soils solidifies a prioritized strategy of root developmental formation under NaCl stress conditions. This mechanism may not be a straight one for immediate restoration of salinity costs but it seems to be adequately resilient for survival under continuous stress, potentially via salt compartmentation and exclusion as it is observed in other tree species [58]. Similar results in saline stress conditions have been reported for *Citrus* species, olives and numerous vegetable and field crops [59–61].

In orchard soil saline conditions, phenolics play a protective role against stress-related reactive oxygens species (ROS), osmotic and ionic damages [62]. *P. spinosa* saline unchallenged plantlets and parentally survived in the wild provided total phenolic content similar to this work in the Lagadas, Pieria and Chalkidi areas of northern Greece [29]. Values of total phenolic content in leaves did not change among soil treatments and salinity levels of applied stress. A similar total phenolics data trend under the same scaling stress factor was documented for new leaves in salt-sensitive Leccino var. olive trees and Brassicaceae plants [63,64]. In contrast, incremental changes in total phenolics were found in leaves of four olive tree varieties with content pick at 125 mM NaCl saline stress [65]. For commercially valuable pear species, e.g., *Pyrus communis*, total phenolic high/low reaching concentrations are dependent to genetics, leaf age and environmental stress existence [66]. Grafted and ungrafted *P. communis* seedlings under drought stress, a physiological condition comparable to saline stress as used here, did not drastically change their total phenolic

content through scaled up stress (moderate, severe), reaching a value plateau [67]. Saline soil effect, alleviated via l-methionine and microbial enrichment, did not change total phenolics concentration; however, amino acid foliar application on saline-stressed plants increased their total phenolic content, whereas field microbial support is capable of such upregulation if exogenous microorganisms dominate on native soil microbial flora [48,68].

Three-year-old *P. spinosa* seedlings decline their height growth rates in exposure to NaCl after 30 days; treatments with 75 mM and 150 mM salt concentrations conferred 28.9% and 43% inhibition effect, respectively [69]. In this work, three-month-old *P. spinosa* seedlings' height growth rates declined in the presence of soil sodium chloride, which may due to a) low ability to cell elongation, b) suboptimal-to-detrimental osmotic regulation and c) counter growth hormonal status [70]. Additionally, month by month data analysis (April–July) of seedling height growth responses interfered also with temperature increase and rainfall decline in addition to salt stress and/or exogenous treatments [15,71,72].

Data of l-methionine supplementation provided resilient aspects in saline stress conditions; however, amino acid soil application in this work cannot be directly correlated to potential photosynthetic enhancement due to non-foliar administration, as it is shown in other experimental conditions [19,73]. Exogenous bacterial rhizosphere depositions improved growth traits in accordance with other tree species, such as olives and bananas, where microorganisms offer photo-oxidative protectant and photosynthetic advancement roles [26,74].

Under saline conditions, *P. spinosa* exhibits higher photosynthetic rates with lower Na and Cl ion concentrations in leaves in comparison to native Asian pear species, *P. betulaefolia*, *P. pyrifolia* and *P. xerophila*, emerging as a protective pattern for chlorophyll functionality as observed to other plants [69,75]. Herein, leaf chlorophyll index reduced gradually when salinity concentration increased for all treatment lines. This attribute was also shown in olive trees where stress was mitigated by foliar use of gibberellic acid [76]. Exogenous foliar application of methionine on C4 plants (maize) ameliorated the content of chlorophylls; however, this alleviation effect was not achieved in our C3 *P. spinosa* plants [48]. In comparison to *P. spinosa*, maize as a C4 plant with NADP-dependent malic enzyme (NADP-ME) and phosphoenolpyruvate carboxykinase (PCK) type of photosynthetic capacity provides within bundle sheath and mesophyll cells a developmentally and functionally resilient grana physiology under saline conditions [77]. Plateau chlorophyll index values of 75 mM and 100 mM NaCl saline stress for—all to none—soil enrichments have an underlying more toxic effect of the pressure factor due to plantlet potential failure in osmotic adjustment, regulation of ion homeostasis and cytoplasm metabolism inhibition [78].

Antioxidant capacity found in *P. spinosa* leaves was increased in saline environment, reaching plateau in 75 mM/100 mM NaCl soil stress; however, this phenomenon took place as a non-specific one towards these two types of diverse basis (biochemical and microbial) soil enrichments. Many tree species like olives and *Moringa oleifera* advance their antioxidant capacity when exposed to saline [65,79]. Advanced antioxidant activity in *Pyrus* species under saline conditions was recorded in exogenous administration of sodium nitroprusside as nitro oxide donor in *P. communis* [80].

No differences in root orientation (angles 30°–90°) and their frequencies were observed for soil saline exposure of *P. spinosa*, independently of exogenous l-methionine and bacterial alleviation treatment; this suggests low levels of root architectural plasticity under salt stress, which is not commonly observed in resilient and halophytic plants due to advance hormonal management [81–83]. L-methionine-supplemented seedlings on NaCl-free soil increased total root length, a response that is also reported in other horticultural species [84]. However, it should be taken into account that saline soil integration of methionine did not always confer a counteract salinity effect; this may also be due to occurring changes in its thermodynamic properties and solubility in the presence of NaCl [85,86]. Bacterial supplementation supported root expansion in length as recorded in other plant species due to positive spatial interaction between plant host and microorganisms [55].

Supplementation of bacterial complex population (*Bacillus subtilis*, *B. pumillus*, *B. licheniformis*, *B. megaterium*, *Azotobacter* sp.) promoted *P. spinosa* survival strategy via improved stress root management. *Bacillus subtilis*, *B. pumilus*, *B. licheniformis* and *B. megaterium* salinity alleviation properties were reported extensively for field crops [57,87–91] and vegetable crops [92–95]; however, research data for tree species are still few. Limitations for sustainable use of *B. subtilis* as soil orchard inoculant exist due to low iron solubility and downregulation of iron-related genes in NaCl-enriched environments [96]. *Azotobacter* sp., a root colonized with biofilm formation attribute bacterial species, provided contributory alleviation support to saline stress; however, most of the comparably documented work was performed on annual plant species where root–shoot hardiness and tissue development differ from perennial ones [97–99].

Plant species with parental growth in coastal wild conditions like *P. spinosa* are possible to carry extensive intergenerational and transgenerational stress memory due to continuous soil and air droplet saline challenge status; thus, examination of resilience mechanisms may not be too apparent and distinctive due to pre-conditioned abiotic exposure [16,100]. Therefore, it is not known if minimal to non-significant response differences found among treatments on *P. spinosa* seedlings were due to preformed salinity stress memory [16,101,102]. Salinity stress-related memory for tree species in agricultural practice has been reported for olives [103].

5. Conclusions

Young *P. spinosa* plantlets are capable to grow in less than 100 mM NaCl soil conditions with scaled up temperatures (April–July) and low seasonal moisture availability. Alleviation saline stress biochemical and microbial tools, which were applied to minimize physiological and nutritional deterioration, appeared to have tissue-specific effects with emphasis on root expansion for survival. Leaf chlorophyll, total phenolic content and antioxidant activity appeared to follow the same trends independently of soil enrichment with formulated sets of l-methionine and bacterial inoculants. Root orientation did not change under saline stress with or without the above mentioned supplementation.

Further work is needed to (a) analyze anatomical changes in *P. spinosa* seedlings under saline stress jointly with l-methionine and bacteria presence, (b) investigate transcriptional changes in induced *P. spinosa* root expansion survival strategy when supplemented with l-methionine and bacteria, (c) examine contributory aspects of salinity stress memory and (d) explore age-related responses to saline stress.

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