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Use of microbial biostimulants to enhance the salinity tolerance of tomato transplants ⁺

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Abstract: Vegetable plants are more sensitive to salt stress during the early growth stages, hence, the availability of poor-quality brackish water can be a big issue for the nursery vegetable industry. Microbial biostimulants may promote growth and vigor and counterbalance salt stress in mature plants. This study aimed to evaluate the application of plant growth-promoting microorganisms for improving salt tolerance of lettuce and tomato seedlings irrigated with increasing salinity (0, 25, and 50 mM NaCl) during nursery growth. Two commercial microbial biostimulants were applied to the substrate before seeding: 1.5 g L-1 of TNC Bactorr^{S13} containing Bacillus spp.; 0.75 g L-1 of Flortis Micorrize containing Glomus spp., Agrobacterium radiobacter, Bacillus subtilis, Streptomyces spp. and Thricoderma spp.. Lettuce and tomato seedlings suffered negative effects of salinity on many morpho-physiological parameters. The use of the microbial biostimulants modified seedling growth and its response to salt stress. They had a growth-promoting effect on the unstressed seedlings increasing fresh and dry biomass accumulation, leaf number, and leaf area and were successful in increasing salinity tolerance of seedlings especially when using Flortis Micorize that enhanced salinity tolerance up to 50 mM NaCl. The inoculation of the substrate with microbial biostimulants could represent a sustainable way to improve tomato transplant quality and to use brackish water in vegetable nurseries limiting its negative effect on seedling growth.

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Copyright: © 2021 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/). **Keywords:** vegetable crops; nursery; seedling growth; salt stress; PGPR; arbuscular mycorrhizal fungi; *Trichoderma*

1. Introduction

Plant sensitivity to salt stress is generally higher at earlier growth stages (seedling, establishment) than at later stages [1]. Thus, growing seedlings under conditions of scarcity of good quality water is a difficult challenge for the nursery vegetable industry. Vegetable transplant growers aim to obtain well-developed and vigorous seedlings that can establish and grow fast after transplanting [2,3]. The negative effects of irrigation water salinity on seedling growth can make hard for vegetable nursery to reach these goals.

The rebalancing of phytohormones levels through the exogenous application of phytohormones has been suggested as a strategy to increase salt tolerance of vegetable crops and mitigate the negative effects of salinity [4]. This rebalancing can be achieved by direct supplementation of synthetic plant growth regulators through foliar or root supplementation [5,6] or by inoculating the rhizosphere with microorganisms, which produce phytohormones or interact with plants, inducing hormonal changes. The use of microbial inoculants is becoming a more widely accepted technique for improving the sustainability of intensive agriculture systems. Microbial inoculants, also referred to as microbial bi-

MDPI

ostimulants, such as plant growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi (AMF) and *Trichoderma* spp. are considered useful tools to mitigate the effects of salinity on plant growth and yield. Microbial biostimulants have been applied to many vegetable crops to improve plant growth and stress tolerance but they have not been yet applied to vegetable transplant production. Therefore, this study aimed to evaluate the use of microbial biostimulants to increase salt tolerance of tomato seedlings during nursery growth.

2. Materials and Methods

A nursery trial was carried out in a greenhouse situated at the Department of Agricultural, Food, and Forest Sciences (SAAF-University of Palermo, Italy) (38°6'28" N 13°21'3" E; altitude 49 m above sea level) to evaluate the effects of salt stress and microbial biostimulant inoculation on transplant production. During autumn 2020, seeds of Solanum lycopersicum 'Marmande' (Vilmorin, La Ménitré, France) were sown into 9 polystyrene trays with 104 cells each. Three trays were filled with a commercial substrate (Utilis, GreenView srl, Crocetta del Montello, Italy, fertilized with 850 g m⁻³ of a mineral fertilizer NPK), other three trays were filled with the same substrate inoculated with 1.5 g L^{-1} of TNC Bactorr^{S13} (The Nutrient Company, Rochdale, UK), and the remaining trays were also filled with the commercial substrate inoculated with 0.75 g L⁻¹ of Flortis Micorrize (Orvital, Settimo Milanese, Italy). Bactorr^{S13} (B) and Flortis Micorrize (M) are commercial biostimulants: the first contains plant growth-promoting bacteria (1.3 108 CFU g⁻¹ of Bacillus amyloliquefaciens, B. brevis, B. circulans, B. coagulans, B. firmus, B. halodenitrificans, B. laterosporus, B. licheniformis, B. megaterium, B. mycoides, B. pasteurii, B. subtilis, and Paenibacillus polymyxa) as well as soluble humates, natural plant hormones. amino acids, vitamins, and trace elements derived from Ascophylum nodosum; the second contains 30 % of Glomus spp., 1.24 x 10⁸ CFU g⁻¹ of Agrobacterium radiobacter, Bacillus subtilis, Streptomyces spp. and 3 x 10⁵ CFU g⁻¹ of *Thricoderma* spp..

After sowing (October 2020), the trays were kept in a dark room at 22 ± 1 °C and were transferred to the greenhouse for seedling growth when the first emergence was observed (4 days after sowing).

At tomato seedlings first true leaf, salt treatments were applied with an ebb and flow sub-irrigation system using water with 0 mM NaCl (Electrical conductivity - EC 0.68 mS cm⁻¹), 25 mM NaCl (EC 3.14 mS cm⁻¹) or 50 mM NaCl (EC 5.57 mS cm⁻¹). Seedlings were sub-irrigated according to their need until they were ready for transplanting (twice a week on average) and sub-fertigated once a week with 3 g L⁻¹ of a water-soluble NK fertilizer (13-46). The trays were weighed individually before and after each sub-irrigation to measure the water consumed during growth and calculate the water use efficiency (WUE) as WUE (g DW L⁻¹ H₂O) = plant dry weight (g)/H₂O (L). One week before the end of the experiment, leaf stomatal conductance was measured using a diffusion porometer (AP4, Delta-T Devices Ltd., Cambridge, England) on two young unshaded leaves of 20 seedlings for each replicate of each combination biostimulants x salt stress.

The seedlings were considered ready for transplanting when they had 4-5 true leaves (28 days from sowing). At that time, transplants were randomly selected (four replicated samples of 30 seedlings for each treatment) and their morphological characteristics (seedling height, stem diameter, and leaf number) were evaluated. Leaf color was measured on the upper part of 2 randomly selected leaves of each seedling with a colorimeter (CR-400, Minolta corporation, Ltd., Osaka, Japan) that recorded CIELAB parameters (L*, a* and b*). Hue angle (h°) and Chroma (C*) were calculated from a* and b* values as h° = 180° + $\arctan(b^*/a^*)$ and C* = $(a^{*2} + b^{*2})^{1/2}$. Soon after, leaves, stem, and roots were separately weighed and dried at 85 °C to a constant weight to calculate the fresh and dry biomass and the shoot/root ratio for both fresh and dry weight. Before drying, the leaf area of each seedling was measured by scanning leaves at 350 dpi (Epson Perfection 4180 Photo, Seiko Epson Corp., Suwa, Japan) and analyzing the digital images obtained with the ImageJ 1.52a software (National Institutes Health, Bethesda, MD, USA). Leaf area and leaf dry

weight were used to calculate the specific leaf area (SLA $cm^2 g^{-1} DW$) as SLA = leaf area/leaf dry weight.

At the end of the nursery trial, the water status of the seedlings of each treatment was assessed by determining their relative water content (RWC). Ten leaves for each replicate were randomly sampled and their fresh weight was immediately measured; then, leaves were placed in distilled water for 4 h to measure their turgid weight (TW) before drying in an oven at 80 °C for 24 h. Dried leaves were weighed (DW) and finally, the relative water content was calculated as RWC = $(FW - DW)/(TW - DW) \times 100$.

The experimental design consisted of four replicated samples of 30 seedlings each for every combination of microbial biostimulant and NaCl level, randomly assigned in four blocks. A two-way ANOVA was used to analyze the effects of microbial biostimulants and NaCl levels on tomato seedlings. The mean values of each parameter considered were compared by the least significant differences (LSD) test at $P \le 0.05$ to discriminate the differences among treatments and the interactions between factors.

3. Results

Tomato plantlets emerged 4 days after sowing and seedlings were ready for transplanting (4–5 true leaves; 14–15th BBCH growth stage) after 28 d from sowing. The height of tomato seedlings was significantly affected only by the salt stress. It linearly dropped from 21.0 cm to 15.3 cm as salt stress increased (Table 1).

Table 1. Effects of microbial biostimulant treatment (C, not treated; B, Bactor^{S13}; M, Micorrize) and salt stress (0, 25, and 50 mM NaCl in the irrigation water) on morphological parameters of tomato seedlings.

Source of Vari- ance		Seedling Height - (cm)	Seedling fresh weight (g FW)				Seedling dry weight (mg DW)				_Dry mat-	WUE (g DW L-1
			Total	Roots	Stem	Leaves	Total	Roots	Stem	Leaves	ter (%)	DW L ⁻¹ H ₂ O)
Treatment												<u> </u>
С		^z 18,1	2,44	0,30b	1,31	0,82	184,4c	26,4c	75,6c	82,3b	7,5c	3,5
В		18,3	2,63	0,32ab	1,38	0,93	213,3b	28,0b	87,7b	97,6a	8,1b	3,9
М		18,9	2,64	0,33a	1,41	0,91	224,7a	29,3a	93,2a	102,2a	8,6a	4,0
NaCl (mM)												
0		21,0a	2,93	0,32a	1,64	0,97	218,4a	29,8a	92,0a	96,6a	7,2c	3,6
25		19,1b	2,69	0,34a	1,44	0,91	216,0a	28,2a	91,6a	96,2a	8,0b	4,1
50		15,3c	2,10	0,29b	1,03	0,78	187,9b	25,8b	72,8b	89,3b	9,0a	3,7
Treatment × NaCl												
С	0	20,2	2,73bc	0,31	1,53b	0,89bc	195,6	29,3	79,8	86,5	6,9	3,4c
	25	19,2	2,53c	0,31	1,40bc	0,82c	188,9	26,7	81,0	81,3	7,3	3,5c
	50	15,0	2,05d	0,29	1,01d	0,76c	168,6	23,3	66,0	79,3	8,2	3,5c
В	0	20,9	3,11a	0,34	1,72a	1,05a	228,6	29,3	97,0	102,3	7,2	4,0b
	25	18,6	2,66c	0,33	1,39c	0,94b	216,9	28,7	90,3	98,0	8,1	4,1b
	50	15,6	2,13d	0,29	1,05d	0,79c	194,3	26,0	75,8	92,5	9,1	3,8c
М	0	21,9	2,96ab	0,32	1,67a	0,97b	230,9	30,7	99,3	101,0	7,6	3,6c
	25	19,6	2,86b	0,36	1,52b	0,98b	242,1	29,3	103,5	109,3	8,5	4,6a
	50	15,4	2,11d	0,31	1,02d	0,78c	201,0	28,0	76,8	96,3	9,6	4,0b
Significa	ance ^x											
Treatment		ns	***	**	**	***	***	*	***	***	***	***
NaCl		***	***	***	***	***	***	**	***	*	***	***
Treatment × NaCl		ns	***	ns	*	***	ns	ns	ns	ns	ns	***

² Each value is the mean of 4 replicated samples of 30 seedlings each. For each factor, values in a column followed by the same letter are not significantly different, according to the least significant differences (LSD) test. × Significance: ns = not significant; * significant at p < 0.05; ** significant at p < 0.01; *** significant at p < 0.001. WUE: water use efficiency.

Seedling total fresh weight (FW) was 2.73 g in the unstressed control seedlings and was reduced significantly (-24.8%) with 50 mM NaCl. The seedlings inoculated with Bactor^{S13} (B) and Micorrize (M) had a higher total fresh weight than control seedlings under no salt stress (3.11 and 2.96 g, respectively) (Table 1). Under moderate and high stress, B-seedlings exhibited a reduction of total fresh biomass, close to those of control seedlings (2.66 and 2.13 g with 25 and 50 mM NaCl, respectively), whereas M-seedlings did not

suffer a significant reduction of total fresh weight with 25 mM NaCl (2.86 g) that was significantly higher than that of control seedlings under the same stress level (+12.9%). The lower root fresh weight was recorded in control seedlings and the hypogeal part of the seedling was significantly increased by M biostimulant (+8.8%). All the seedlings suffered a reduction of root fresh weight under the highest salt stress, irrespective of the biostimulant treatment. The modifications of stem fresh weight were similar to those reported for the total fresh weight. The fresh weight of the leaves of the unstressed seedlings was higher in those inoculated with B (1.05 g) than those of M treatment (0.97 g) and control (0.89 g). The highest salt stress reduced leaf fresh weight down to 0.78 g on average in all the treatment, whereas the seedling inoculated with the microbial biostimulants had a significantly higher leaf fresh biomass than control under the intermediate salinity level (0.96 g on average for B and M, +17.4% than control). The dry weight (DW) of the seedlings and all the seedling parts (roots, stem, and leaves) was negatively affected by salt stress only at the highest salinity level, irrespective of biostimulant treatments. The microbial inoculants were effective in increasing total dry biomass accumulation. The B-treated seedlings had higher total dry biomass than control, whereas the M-treated seedlings accumulated significantly more dry biomass than B-treated and control seedlings. The same differences were recorded also for root and stem dry weight, while the leaf dry weight of the seedlings inoculated with B and M showed no differences among them and was significantly higher than control (+21.3% on average) (Table 1). The dry matter percentage was 7.5% in control seedlings and raised to 8.1% in the seedlings inoculated with B and even more inoculating the substrate with M (8.6%) (Table 1).

The water use efficiency (WUE) was not affected by salt stress in control seedlings that accumulated 3.5 g DW L⁻¹ H₂O. The seedling inoculated with B had a higher WUE than control with 0 and 25 mM NaCl. The seedling inoculated with M showed the highest WUE with 25 mM NaCl (4.6 g DW L⁻¹ H₂O) and had a higher WUE than control also with 50 mM NaCl (Table 1).

Table 2. Effects of microbial biostimulant treatment (C, not treated; B, Bactor^{S13}; M, Micorrize) and salt stress (0, 25, and 50 mM NaCl in the irrigation water) on the leaf characteristics of tomato seedlings.

Source of Variance		Number of Leaves	Leaf Area (cm ² Seedling ⁻¹)	SLA ^y (cm ² g DW ⁻¹)	Stomatal Conductance (mmol m ² s ⁻¹)	RWC (%)	L*	Chroma	Hue°
Treatm	nent								
С		^z 4.9b	44.5	540.2a	392.2	84.3	45.1	30.2	127.6
В		5.2a	49.2	503.7b	454.4	89.5	45.0	30.1	127.1
М		5.1a	50.4	493.5b	358.1	90.3	45.1	30.4	127.4
NaCl (1	mM)								
0		5.2a	52.7	547.1a	545.4	92.1	45.4a	30.8a	127.2
25		5.1a	49.9	523.8b	358.0	89.9	45.3a	30.6a	127.2
50		4.9b	41.4	466.4c	301.2	82.1	44.5b	29.3b	127.7
Treatment \times NaCl									
С	0	4.9	48.0b	554.8	389.7b	92.6a	45.9	31.4	127.0
	25	4.9	46.1b	568.7	385.0b	90.6b	45.3	30.1	127.6
	50	4.9	39.4c	497.2	401.8b	69.7c	44.1	29.0	128.1
В	0	5.4	55.9a	548.9	759.8a	90.8ab	45.4	30.5	127.1
	25	5.2	49.4b	504.2	354.4b	88.9b	45.0	30.2	127.2
	50	5.0	42.4c	458.1	249.0b	88.8b	44.7	29.6	127.1
М	0	5.2	54.2a	537.7	486.8ab	93.0a	45.1	30.4	127.5
	25	5.2	54.4a	498.6	334.7b	90.1ab	45.7	31.5	126.9
	50	4.8	42.6c	444.1	252.8b	87.8b	44.5	29.3	127.8
Signific	ance ^x								
Treatment		**	***	***	ns	**	ns	ns	ns
NaCl		**	***	***	***	***	***	**	ns
$Treatment \times NaC$		ns	**	ns	**	***	ns	ns	ns

^z Each value is the mean of 4 replicated samples of 30 seedlings each. For each factor, values in a column followed by the same letter are not significantly different, according to the LSD test. × Significance: ns = not significant; * significant at p < 0.05; ** significant at p < 0.01; *** significant at p < 0.001; y Specific leaf area. RWC: relative water content.

The seedlings inoculated with microbial biostimulants recorded the highest leaf number (Table 2). The number of leaves significantly decreased only at the highest concentration of NaCl in the irrigation water (-5.5% with 50mM NaCl than control). The total leaf area of seedlings was positively affected by microbial biostimulants (+14.8% on average than control). With 25 and 50 mM NaCl, the total leaf area of control seedlings did not differ significantly from B-treated seedlings, whereas M-treated seedlings were not affected by the moderate salt stress and reduced their total leaf area only with 50 mM NaCl (Table 2). The specific leaf area (SLA) was lower in the seedling treated with microbial biostimulants and decreased as salt stress increased. The unstressed seedlings had a higher stomatal conductance when inoculated with the microbial biostimulants treatment but recorded a reduction of this parameter under salt stress (Table 2). The relative water content (RWC) was significantly lowered at each salt stress level in control seedlings (from 92.6 to 69.7%), while the seedlings inoculated with biostimulants reduced their RWC with 25 mM NaCl to values similar to those recorded in the control with no further significant decrease at the highest salinity level (Table 2). Leaf color was affected only by irrigation water salinity. The highest salt stress level increased leaf color lightness (L*) and reduced its vividness, as showed by the lower value of Chroma (Table 2)

4. Discussion

Uninoculated tomato seedlings irrigated with saline water suffered a decrease of biomass accumulation and growth limitations. Many vegetable crops suffer similar alteration when grown under salt stress in open field or nursery [7,8]. The use of brackish water for seedling irrigation affected the total fresh and dry biomass of the untreated seedlings. Total fresh and dry weights were significantly affected only at the highest salinity level with a reduction of -24.8% and -13.8% with 50 mM NaCl, respectively. Soil or irrigation water salinity affect plant growth and its metabolism in many different ways. The negative effects of salt stress may slow down or even stop plant growth, change biomass partitioning and plant morphology [9], as shown by the variations in biomass accumulation, fresh weight shoot/root ratios and reduction of leaf number and leaf expansion in tomato seedlings. Growth limitation due to salinity can reduce the size of transplants which is linked to establishment success, the growth rate, and the size at harvest [10], thus decreasing the commercial success of these products.

The inoculation of the substrate with microbial biostimulants exerted a growth-promoting effect on the unstressed tomato seedlings but this effect differed between B and M. The use of microbial biostimulants was also effective in modifying the tolerance of seedlings to salt stress. Treatments with B and M delayed the beginning of salt stress symptoms and limited growth reduction of tomato seedlings at the intermediate salinity level (25 mM NaCl), resulting in fresh biomass accumulation similar to those of the unstressed untreated tomato seedlings and higher dry biomass and leaf area especially in salt-stressed tomato seedlings inoculated with M. An improvement of water use efficiency was recorded in the salt-stressed inoculated seedlings, but the effect of M was almost twice than B in both salinity treatments. Microbes can be involved directly or indirectly with plant growth promotion, either through improved nutrient acquisition and hormonal stimulation or the suppression of plant pathogens resulting in more vigorous and healthier plants [11]. Several microorganisms that can promote plant growth are well-studied in their mode of action and regulation, and comprise members of bacterial (Azospirillum, Bacillus, Pseudomonas, Rhizobium, Serratia, Stenotrophomonas, and Streptomyces) or fungal (Ampelomyces, Coniothyrium, and Trichoderma) genera [11]. Besides, arbuscular mycorrhizal (AM) fungi are the most widespread root fungal symbionts and have a role in the stimulation of plant growth and nutrient uptake of many host plants [12]. It is well known that many Bacillus species can exert a plant growth-promoting effect [13] and the inoculation of the substrate with *Bacillus* spp. promoted seedling growth and biomass accumulation in lettuce transplants [3]. Enhanced growth can be also obtained by inoculating the soil with mycorrhizal fungi [14,15]. The use of microbial biostimulants can also mitigate salinity effects on vegetables. The inoculation of plants of various crop plants with bacteria or AM fungi can result in more vigorous plants and enhanced tolerance to salt stress [16,17].

The initial inoculation of the substrate with microbial biostimulants was successful in enhancing plant growth and allowed to increase the salinity tolerance, especially when using the biostimulant that was characterized by the highest biodiversity as it contained both bacteria (*Agrobacterium radiobacter, Bacillus subtilis, Streptomyces* spp.) and fungi (*Glomus* spp. and *Thricoderma* spp.).

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