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Biostimulation effect on *Crithmum maritimum* L. root development in controlled environments

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Abstract: Biostimulants can be used in sustainable agriculture as they are regarded as inputs with minimal environmental impact. When applied to the root systems of certain plant species, microbial inoculants confer physiological and morphological benefits to the entire plant. In the present study, we focused on some morphometric parameters of the root system of two *Crithmum maritimum* L. spontaneous populations (Mediterranean and Atlantic). We compared the effects of two different (for number of strains) biostimulants on the weight, length, diameter, and number of tips on the root system. The experiment was conducted in two controlled environments: i) an incubator in which seeds were placed in Petri dishes on Whatman filter paper and ii) a greenhouse where seeds were placed in pots containing seeding substrate. Under incubator conditions, the biostimulant application showed, on average, a stimulating effect on the analyzed root parameters. Specifically, it increased weight by approximately 85%, diameter by 25%, and length by about 128% compared to the control. Similarly, under greenhouse conditions, the biostimulant application showed favorable effects on the analyzed root variables, exhibiting an increase in root weight by approximately 66%, diameter by 25%, length by about 75%, and number of tips by 56% compared to the control. The present study suggests the potential use of biostimulants during the nursery phases of production to ease the establishment of alternative crops such as *Crithmum maritimum* in cropping systems for which it is necessary to implement the agronomic technique and encourage rapid colonization in contexts otherwise at risk of desertification.

Key words: Microbial inoculants, plant growth-promoting rhizobacteria, sea fennel, root parameters

Acronyms:
ME = Mediterranean population;
AT = Atlantic population;
CT = control treatment;
BS1 = biostimulant composition *Azospirillum brasilense*, *Priestia megaterium* var. phosphaticum, *Bacillus circulans*;
BS2 = biostimulant composition *Azotobacter chroococcum* plus, *Azospirillum brasilense*, *Priestia megaterium* var. phosphaticum, *Bacillus circulans*.

1. Introduction

Recently, biostimulants based on live microorganisms have gained significant attention from industry and academia mainly because the growth and development of a plant can be enhanced more easily in the field (Yao et al., 2023). Biostimulants can reduce the agricultural chemical footprint owing to their beneficial multilevel properties that help make agriculture more sustainable and resilient (Koli et al., 2019). When applied to plants or the soil, increased absorption and distribution of nutrients, tolerance to environmental stress, and improved quality of plant products explain the mechanisms by which these probiotics are useful (Mandal et al., 2022). It is also worth noting that microbial-based plant biostimulants, such as those involving plant growth-promoting rhizobacteria (PGPR) from the *Bacillus* and *Pseudomonas* genera (Hashem et al., 2019), nitrogen-fixing *Azotobacter* (Gauri et al., 2012; Wichard et al., 2009), *Azospirillum* (Amavizca et al., 2017; Marques et al., 2021), and the *Rhizobium* species (Santini et al., 2021), make up less than 25% of the commercial biostimulants available on the international market. PGPR helps plants tolerate environmental stress and influence growth and yield through their metabolic activities and the multiple factors generated by this interaction with plants. Thus, inoculation with PGPR leads to economic and environmental gains, which are important for the sustainable intensification of production systems (Barbosa et al., 2022) and notable in organic farming

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systems (Bertrand et al., 2021). In these environmental contexts, *Crithmum maritimum*, also known as sea fennel, has received increasing attention in Mediterranean areas (Zenobi et al., 2021, 2022). This perennial halophyte thrives on sandy beaches, maritime rocks, breakwaters, and piers along coastlines worldwide and is particularly abundant in Mediterranean countries (Atia et al., 2011). Sea fennel is rich in various bioactive compounds that have been extensively investigated and found to possess a wide range of positive properties. These properties include antioxidant (Pereira et al., 2017), antibacterial (Jallali et al., 2014), antifungal (Alves-Silva et al., 2020), cytotoxic (Alemán et al., 2019), anticancer (Chen et al., 2021), antiinflammatory (Alemán et al., 2019; Alves-Silva et al., 2020), antimutagenic (Souid et al., 2021), cholinesterase inhibitory (Generalić Mekinić et al., 2018), vasodilatory (Generalić Mekinić et al., 2016), and antiparasitic (Pereira et al., 2021) properties. *C. maritimum*, a halophytic species known for its ability to grow and develop even under water stress conditions (Azeñas et al., 2019), is a promising candidate for the development of extensive green roofs, even in situations characterized by shallow substrates and limited irrigation (Nektarios et al., 2016). As a member of the halophyte group, which includes plants tolerant to saline environments, sea fennel has been identified as a potential crop for biosaline agriculture (Atia et al., 2011; Piatti et al., 2022; Politeo et al., 2023). This suggests its use in implementing more sustainable cropping systems that require fewer inputs while providing multiple services, particularly in addressing climate change issues and preserving agrobiodiversity (Renna, 2018). Indeed, sea fennel can be used in marginal or degraded areas to promote soil desalination, enable agricultural production, recycle nutrients from aquaculture effluents (Buhmann and Papenbrock, 2013), remediate areas polluted by heavy metals, and serve as a source for biorefinery processes (Hulkko et al., 2023). The germination of *C. maritimum* seeds was previously studied under salinity and chemical factors (Meot-Duros and Magné, 2008), while other halophytes were examined for the effects of organic and microbial biostimulants (Jha et al., 2012) on root biomass during their growing phases. In the scientific literature, studies on in vitro seed crops treated with rhizobacterium *Azospirillum brasilense* exist (Méndez-Gómez et al., 2021). Additionally, information is available regarding seeds placed in pots with substrate, including studies involving *Bacillus* ssp. (Araujo et al., 2021) or a combination of microbial strains (Tyagi et al., 2023).

Several studies have shown the effect of a different salinity range on the root (Hamed et al., 2008) and epigeal biomass (Castillo et al., 2022) development of *C. maritimum*. Given the lack of information on the response of *C. maritimum* root system development to biostimulants, this study aims to assess their effect in a controlled environment as a strategy to improve agronomical techniques starting from nursery phases and enhance biodiversity in low-level intensification cropping systems in the Mediterranean environment.

2. Materials and methods

Two experiments were conducted under controlled conditions, one at the incubator level and the other in a greenhouse environment.

2.1. Crithmum maritimum seed collection

In the autumn of 2022, seeds from two populations (Atlantic (AT) and Mediterranean (ME)) were collected at full ripening. The AT population was harvested along the shoreline of western Brittany at the Pointe du Toulinguet (France), while seeds from the ME population were collected along the coastline of the Conero Regional Natural Park in the Marche region (central-eastern coast of Italy). After removing immature seeds and impurities by manual screening, the seeds were stored in a cold room at 5 °C until bacterization.

2.2. Bacterization

Two different biostimulants, labeled BS1 and BS2, were used for the bacterization of the sea fennel seeds and their subsequent biostimulation after sowing. BS1 was composed of three different strains purchased from the international culture collection of the DSMZ[1]. These strains included *Azospirillum brasilense* (strain DSMZ 1690), used for atmospheric nitrogen fixation, *Priestia megaterium* (strain DSMZ 339) as phosphorus-dissolving bacteria, and *Niallia circulans* (strain DSMZ 30598) as potassium-solubilizing bacteria, at a ratio of 2:1:1 (v/v/v). BS2 also consisted of these strains but included *Azotobacter chroococcum* (strain DSM 2286) for nitrogen fixation at a strain ratio of 1:1:1:1 (v/v/v/v) (Abdallah et al., 2021). Both biostimulants were applied to the ME and AT *C. maritimum* populations. The bacterial strains were individually grown in nutrient broth for 48 h at 30 °C in a rotary shaking incubator (SKI 8R, ArgoLab, Carpi, Italy) set at 150 rpm. The biomass was then harvested by centrifugation (Rotofix 32 A, Hettich, Tuttlingen, Germany) at 4000 rpm for 5 min. Following centrifugation, the supernatant was discarded, and the cell pellet was resuspended in 10-mL sterile deionized water. The concentration of bacterial cells was determined spectrophotometrically at 600 nm, using a UV-Vis Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and cell viability was checked using the spread plate method on nutrient agar. Finally, bacterial suspensions of each strain, each containing no fewer than 108 cells/mL, were mixed in the ratios mentioned previously. The seeds were placed in

[1]Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH. Website https://www.dsmz.de/

sterile Petri dishes and pretreated with 10 mL of ethanol (96% solution, v/v) at room temperature to improve seed germination (Corona et al., 2023). After 24 h, the seeds from both populations were rinsed with sterile deionized water and divided into three groups. Two of these groups were treated with BS1 and BS2, while the control group received treatment with sterile deionized water only (Abdallah et al., 2021). Specifically, the seeds in the sterile Petri dishes were treated with 10 mL of BS1 or BS2 and left at room temperature for 3 h under shaking conditions at 100 rpm. Most of the inoculum was then removed using a sterile syringe, and the seeds were left to dry overnight at 30 °C before being sown in pots. Furthermore, 2 L of BS1 or BS2, prepared as described above, was applied once a week to biostimulate the seeds after sowing.

Six treatments were investigated, resulting from the factorial combination of the three biostimulant treatments (control treatment (CT), BS1, and BS2) and the two populations, AT and ME, for each experiment (within an incubator and under greenhouse conditions).

2.3. Experiment description and root development monitoring

2.3.1. Incubator

For the experiment within incubator conditions, Petri dishes containing 20 seeds were prepared, replicated three times for each treatment, and placed inside an incubator. The Petri dishes were then put in a deionized-cooled chamber with a controlled photoperiod. According to Meot-Duros and Magné (2008), there should be a thermoperiod of 20 °C without light. Each set of seeds was placed in tightly sealed 15-cm diameter Petri dishes (20-cm Ø) containing a 125-mm diameter Whatman filter paper (category no. 1441-125) soaked in 1.5 mL of deionized water (CT), 1.5 mL deionized water + 1 mL biostimulator, effectively covering 50% of the surface of the Whatman filter paper. The seeds were inspected daily, and germination was based on visible radical emergence. It is important to note that both BS1 and BS2 concentrations were consistent and administered only when the seeds were placed in the Petri dishes. Daily observations were conducted on the root growth trend after placing the different solutions (CT, BS1, and BS2) on the Whatman paper in the Petri dishes. According to Meot-Duros and Magné (2008), the germination tests for all treatments last 50 days until root elongation occurs (BBCH 6).

2.3.2 Greenhouse

Crithmum maritimum seeds were sown in the third decade of January 2023 and placed on worktables inside a greenhouse; this structure made it possible to maintain an average temperature of 20 °C during the entire winter season. The *C. maritimum* seeds were placed in a seedling nursery tray with 68 round cavities. The hole diameter and diameter of extraction holes were 49 mm and 15 mm, respectively, while the foot height and thickness were 55 mm and 65 mm. These cavities contained a substrate consisting of a mix of topsoil and peat (50:50 ratio), with an electrical conductivity of 0.3 dS m^{-1}, a dry bulk density of 500 kg m^{-3}, a pH (in water) of 6.5, and a total porosity of 85% V V^{-1}. A biostimulant concentration (BS1 and BS2) of 8×10^8 cells mL^{-1} was administered weekly using a manual sprayer. After the third week of March, when the plants had differentiated two true leaves (BBCH 12), they were transplanted into biodegradable pots with an upper diameter and height of 110 mm, resulting in a volume of 8×10^5 mm^3.

2.4. Root parameters acquisition

After 50 days in the Petri dishes in the incubator and at the phenological stages of BBCH scale 13 and 15 (when the plants had differentiated three and five true leaves, respectively) for the pots in the greenhouse, several parameters were measured, including root length, root diameter, number of tips, and the dry weight of root biomass. To analyze the morphology of the root system (root length, diameter, and number of tips), the image analysis system WinRhizo Pro 2007 (Regent Instruments, Sainte-Foy, Quebec, Canada) was used, coupled with a scanner (Epson Expression 10000 XL, Epson America, Inc., Los Alamitos, CA, USA) equipped with an additional light unit (TPU). The root systems were separated from the seeds in the Petri dishes before being analyzed. In the pots, the plants were removed from the substrate and thoroughly washed in tap water multiple times to eliminate all soil residues using a sieve with a 1-mm hole diameter. The root biomass was dried in an oven at 90 °C for 24 h, and its weight was then measured with an analytical scale (Ohaus V14130 Voyager Laboratory Model, Ohaus Corporation, Parsippany, NJ, USA).

2.5. Statistical analysis

Prior to analysis, tests were performed to ensure that normality (Shapiro–Wilk test and QQ-plot) and homoscedasticity assumptions (Levene's test) were met. Each experiment's variables were analyzed using a mixed model, treating Population (P) and Biostimulant (BS) as fixed factors. Replicates and interaction with replicates were considered random factors. In cases of significant differences between the factors ($p < 0.05$), we performed an estimated marginal means post-hoc analysis. To achieve this, the "emmeans" function, with the Bonferroni adjustment from the "emmeans" R package[2], was employed. Notably, a mixed-model analysis was performed separately for the two controlled environments. All statistical analyses were performed using R statistical software (R Core Team, 2019).

[2]Russell, L (2020). Emmeans: Estimated Marginal Means, aka Least-Squares Means, CRAN [online]. Website https://cran.r-project.org/web/packages/emmeans/index.html [accessed 09 December 2023].

3. Results

3.1. Incubator

The results of the mixed model analysis showed significant differences associated with P, BS, and their interaction (P × BS) for almost all measured variables. Specifically, the interaction of the two factors, P × BS, showed differences at $p < 0.001$ for unit dry root biomass, at $p < 0.01$ for root length, and at $p < 0.05$ for root diameter (Table 1).

The analysis of interactions (Figures 1A–1C) revealed that within each biostimulant treatment, statistically significant differences between populations emerged for all three analyzed parameters. Specifically, the BS2 treatment significantly influenced root dry biomass unit weight and root length. In the first case, the ME population recorded significantly higher values (+20%) than AT (Figure 1A).

Conversely, in the case of root length, the AT population exhibited the highest values (+6.7%) (Figure 1B). On the other hand, root diameter was significantly influenced by the BS1 treatment, with statistically higher values observed in the AT population (+3.7%) than in the ME (Figure 1C).

At the population level, a very similar trend was observed. Specifically, for all three parameters, the highest values were recorded for BS2, followed by BS1 and CT, regardless of the population considered (Figures 1A–1C and 2A–2C).

3.2. Greenhouse

Under greenhouse conditions, the results of the mixed model analysis indicated that, at the BBCH 13 phenological phase, root length was significantly affected by both Population and Biostimulant (Table 2). At the Population level, AT exhibited higher values than ME. Concerning the Biostimulant factor, plants treated with BS2 displayed the highest root length. Root dry matter, root diameter, and the number of tips were significantly affected by the P X BS interaction ($p < 0.001$) (Table 2, Figures 3A–3C).

At the five true leaves stage, all four analyzed parameters were highly significantly affected by the P × BS interaction ($p < 0.001$) (Table 2, Figures 4A–4D). The same trend was observed within each population for all analyzed parameters (Figures 5A–5C). Specifically, the values observed in BS2 were significantly higher than BS1 (on

Table 1. Mixed-model analysis of variance containing tests of the fixed effects for Population and Biostimulant under incubator conditions at root elongation phenological phase (BBCH 6).

Factors		**Root dry biomass unit (g)**			**Root length (cm)**			**Root diameter (mm)**		
Population (P)										
Atlantic		0.0016 (±0.0005)			7.00 (±2.73)			0.54 (±0.08)		
Mediterranean		0.0016 (±0.0006)			7.30 (±2.65)			0.54 (±0.09)		
Biostimulant (BS)										
CT		0.0010 (±0.0001)			3.85 (±0.46)			0.46 (±0.06)		
BS1		0.0015 (±0.0001)			7.54 (±0.90)			0.55 (±0.06)		
BS2		0.0022 (±0.0003)			10.06 (±1.05)			0.60 (±0.06)		
	Df	*MS*	*F*	*p*	*MS*	*F*	*P*	*MS*	*F*	*P*
P	1	7.78e-08	9.98	**	2.639	10.62	**	0.002	1.04	n.s.
BS	2	5.80e-07	74.35	***	3.481	14.01	***	0.001	0.51	n.s.
P × BS	2	8.84e-08	11.33	***	1.184	4.77	**	0.006	3.22	*
Residuals	174	7.80e-09			0.248			0.002		

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s.: not significant. Means are followed by standard deviation values.

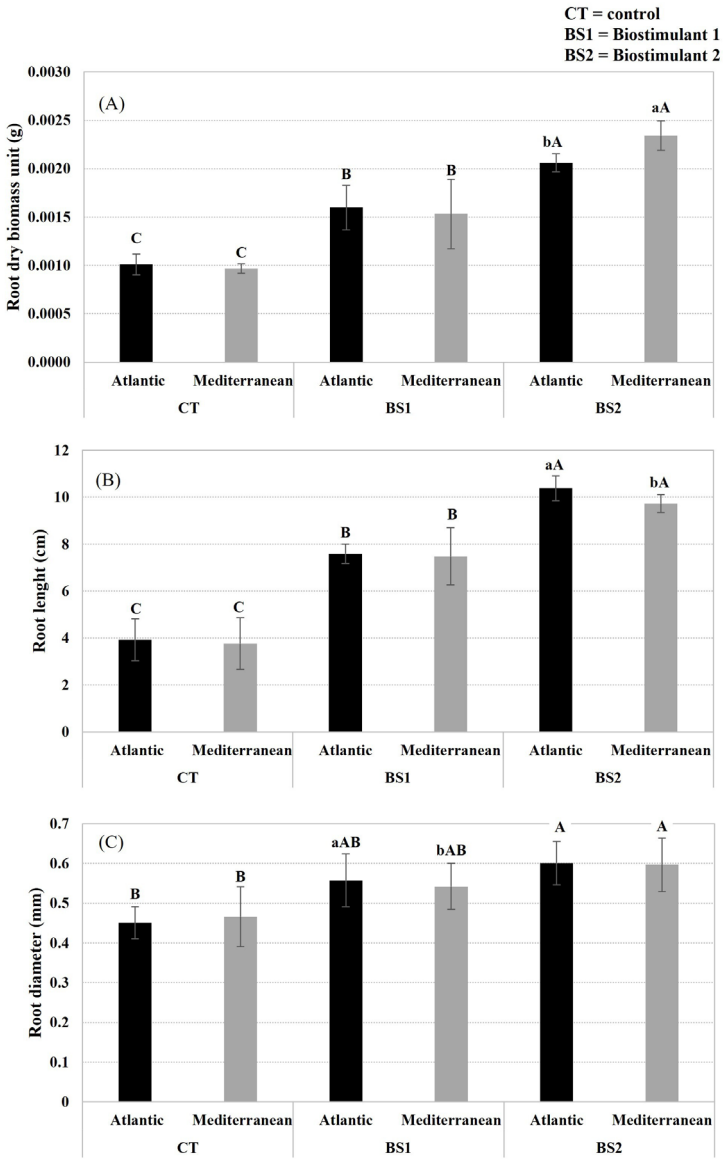

Figure 1. Root dry biomass unit (A), root length (B), and root diameter (C) affected by a significant interaction of Population ´ Biostimulant at the root elongation phenological phase (BBCH 6). Different lowercase letters within each biostimulant treatment mean a significant difference between populations according to estimated marginal means post-hoc analysis; different uppercase letters within each population mean a significant difference among biostimulants treatments according to estimated marginal means posthoc analysis. Bars represent mean ± standard deviation.

average: length: +51%, unit dry biomass: +59%, diameter: +6%, number of tips: +13%) and CT (on average: length: +96%, unit dry biomass: + 128%, diameter: +28%, number of tips: +41%) (Figures 4A–4D).

Within each treatment, differences between populations emerged at the BS1 level in the case of root length (Figure 4A) and at both the BS2 and BS1 level in the case of the number of tips (Figure 4D). Specifically, at the BS2 level, ME showed a higher number of tips than AT (ME, on average, had 259 ± 12 tips vs. 230 ± 11 tips recorded in the AT population). In the case of BS1, the AT population exhibited a higher number of tips than ME, with a total of 220 ± 24 and 213 ± 9 tips, respectively.

Figure 2. Root system in incubator without biostimulant somministration (CT) (A), with Biostimulant 1 (BS1) (B), and Biostimulant 2 (BS2) (C) somministration.

Table 2. Mixed-model analysis of variance containing tests of the fixed effects for Population and Biostimulant under greenhouse conditions during two phenological stages. BBCH 13: phenological phase 3 leaves, and BBCH 15: phenological stage 5 true leaves.

Phenological phase	**Factors**		**Root dry biomass unit (g)**			**Root length (cm)**			**Root diameter (mm)**			**Tips (no)**		
BBCH 13	*Population (P)*													
	Atlantic		0.0024 (±0.00040)			22.00 (±13.26)[a]			0.31 (±0.044)			53.07 (±37.09)		
	Mediterranean		0.0025 (±0.00048)			20.61 (±9.10)[b]			0.31 (±0.049)			49.13 (±31.68)		
	Biostimulant (BS)													
	CT		0.00199 (±0.00002)			10.09 (±2.33)[c]			0.26 (±0.00007)			17.25 (±3.75)		
	BS1		0.00256 (±0.00001)			21.40 (±1.71)[b]			0.32 (±0.005)			50.35 (±7.99)		
	BS2		0.00288 (±0.00011)			32.42 (±3.55)[a]			0.35 (±0.009)			85.70 (±4.10)		
		Df	*MS*	*F*	*p*	*MS*	*F*	*P*	*MS*	*F*	*p*	*MS*	*F*	*p*
	P	1	3.72e-07	27.78	***	226.26	55.84	***	0.0012	8.44	**	4.63	1.68	n.s.
	BS	2	5.92e-07	44.22	***	94.60	23.35	***	0.0065	44.72	***	58.92	21.43	***
	P × BS	2	2.91e-07	21.77	***	2.48	0.61	n.s.	0.0057	39.75	***	28.56	10.38	***
	Residuals	174	1.33e-08			4.051			0.00014			2.74		
BBCH 15	*Population (P)*													
	Atlantic		0.0050 (±0.002)			84.5 (±29.43)			0.33 (±0.04)			207.43 (±30.33)		
	Mediterranean		0.0050 (±0.002)			89.00 (±30.01)			0.33 (±0.04)			215.24 (±42.93)		
	Biostimulant (BS)													
	CT		0.00320 (±0.00005)			61.70 (±1.17)			0.29 (±0.004)			173.08 (±0.31)		
	BS1		0.00460 (±0.00006)			79.01 (±5.44)			0.35 (±0.0003)			216.58 (±4.60)		
	BS2		0.00731 (±0.00408)			119.53 (±2.92)			0.37 (±0.01)			244.34 (±35.92)		
		Df	*MS*	*F*	*P*	*MS*	*F*	*P*	*MS*	*F*	*p*	*MS*	*F*	*p*
	P	1	3.56e-08	0.48	n.s.	173.37	19.81	***	0.0000012	5.59	n.s.	26.40	0.44	n.s.
	BS	2	4.25e-06	57.58	***	572.71	65.46	***	0.0022	19.93	***	333.13	5.61	**
	P × BS	2	1.27e-06	17.28	***	497.56	56.87	***	0.00057	15.23	***	1796.00	30.24	***
	Residuals	174	7.40e-08			8.75			0.000037			59.37		

*p < 0.05, **p < 0.01, ***p < 0.001, n.s.: not significant. Different letters within a column mean significant difference at p < 0.05 level. Means are followed by standard deviation values.

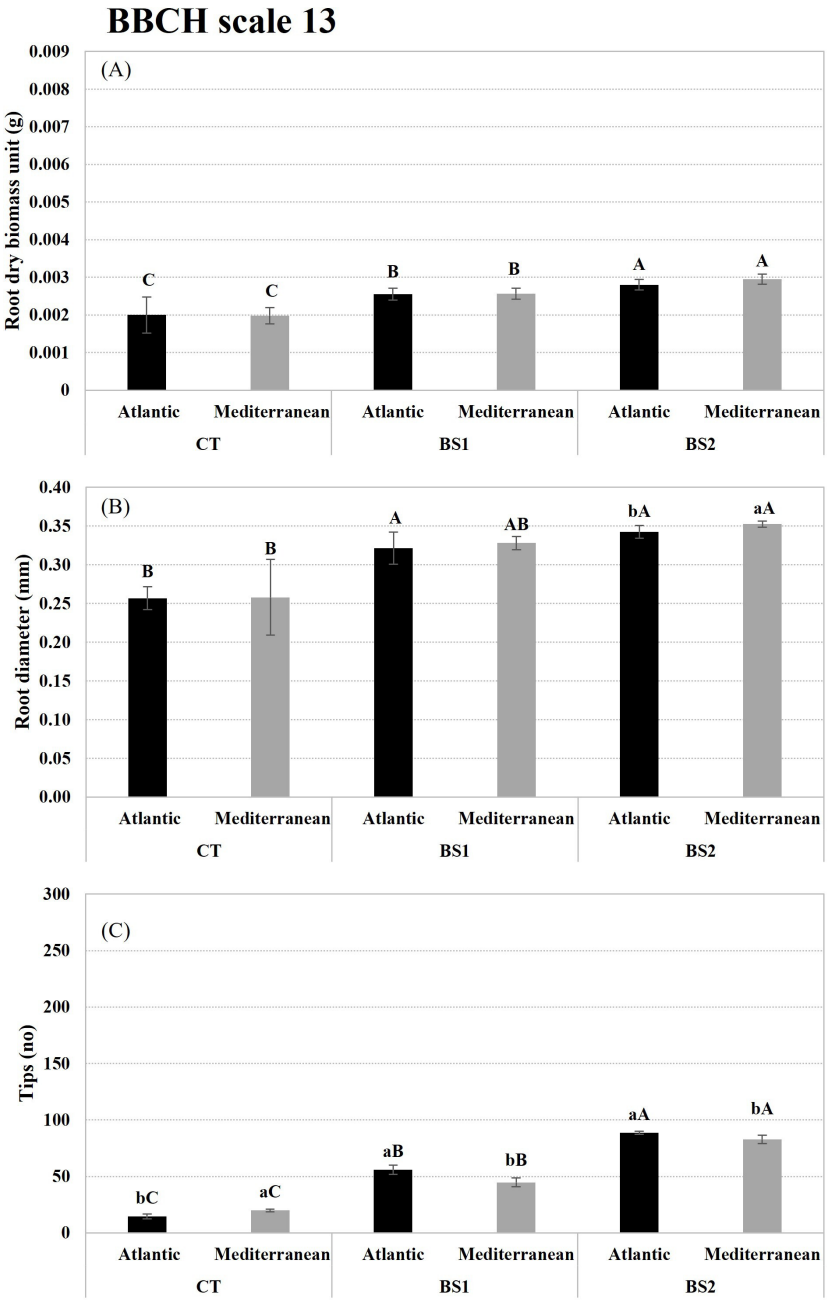

Figure 3. Root dry biomass unit (A), root diameter (B), and number of tips (C) in the compared treatments observed when the plants differentiated three true leaves (BBCH 13). Different lowercase letters within each biostimulant treatment mean a significant difference between populations according to estimated marginal means post-hoc analysis; different uppercase letters within each population represent a significant difference among biostimulants treatments according to estimated marginal means post-hoc analysis. Bars represent mean ± standard deviation.

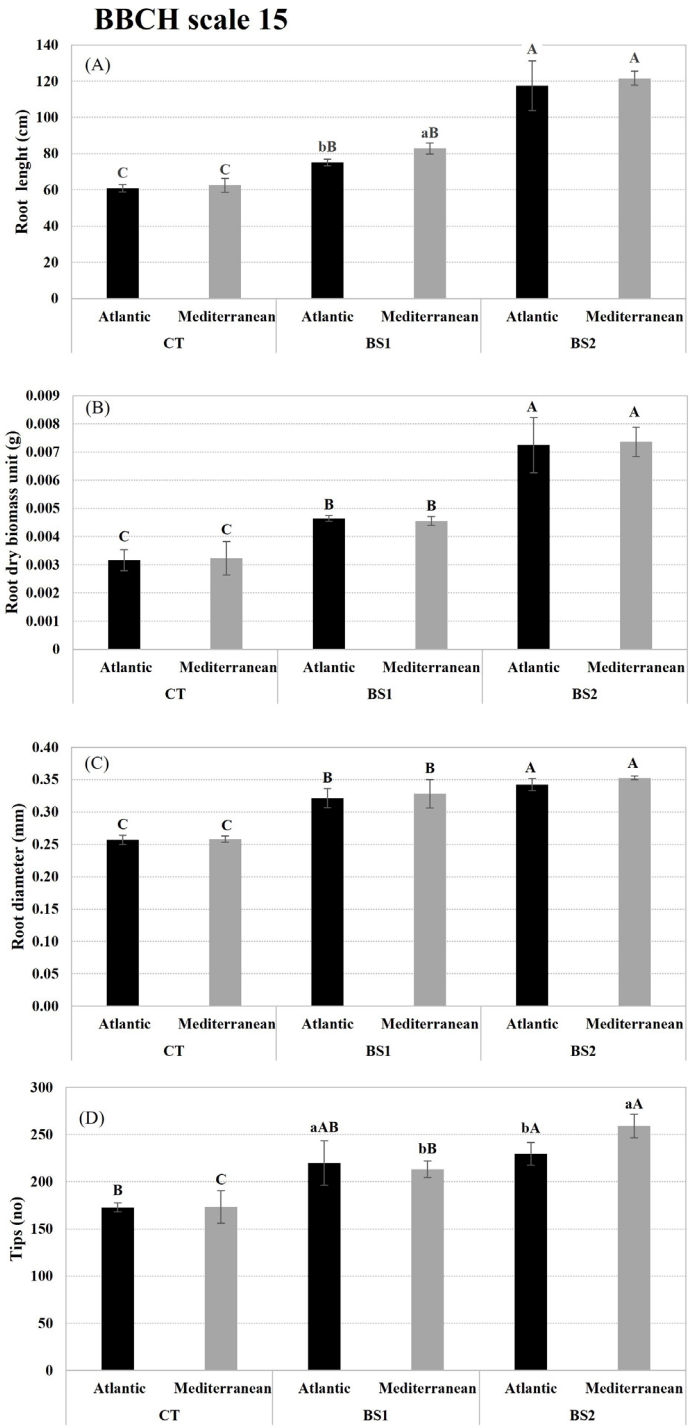

Figure 4. Root length (A), dry biomass unit (B), diameter (C), and number of tips (D) in the compared treatments observed when the plants had differentiated five true leaves (BBCH 15). Different lowercase letters within each biostimulant treatment represent a significant difference between populations according to estimated marginal means post-hoc analysis; different uppercase letters within each population mean a significant difference among biostimulant treatments according to estimated marginal means posthoc analysis. Bars represent mean ± standard deviation.

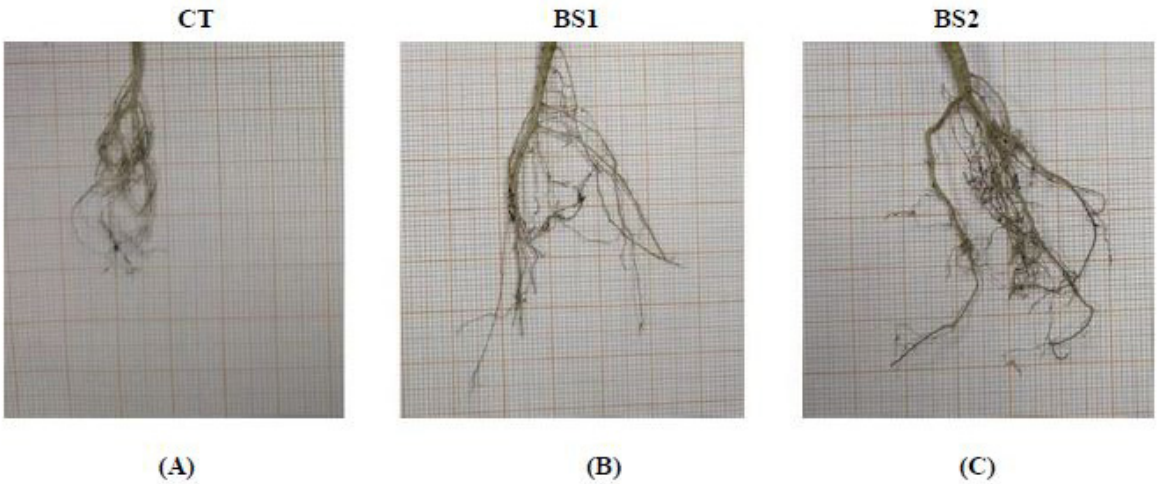

Figure 5. Root system when the plants differentiated five true leaves (BBCH 15): without biostimulant somministration (CT) (A), with Biostimulant 1 (BS1) (B), and Biostimulant 2 (BS2) (C) somministration.

4. Discussion

Within the outlined experimental framework, we analyzed the potential impact of two biostimulants on four key root parameters (root dry biomass, length, diameter, and number of tips) in sea fennel from two spontaneous populations. Microbial biostimulants have gained significant attention in scientific research due to their positive effects on various stages of crop development, spanning from seed germination (Fleming et al., 2019; Yakhin et al., 2017) to plant growth and maturity (Caruso et al., 2019; Colla et al., 2017). Numerous studies have illustrated the benefits of PGPR inoculation across various crops, such as *Crocus sativus* L. (Chamkhi et al., 2023), *Zea mays* L. (Marques et al., 2023), *Foeniculum vulgare* Mill. (Abdallah et al., 2021), *Arabidopsis thaliana* L. (Méndez-Gómez et al., 2021), *Solanum lycopersicum*, L. (Narendra Babu et al., 2015), and *Cicer arietinum* L. (Khan et al., 2018). Previous studies have also explored the effect of salinity on seed germination in Petri dishes for *Crithmum maritimum* (Meot-Duros and Magné, 2008). Research on sea fennel root morphology is limited, primarily due to challenges associated with root sampling and evaluation. However, recent strides in digital image analysis, notably through WinRhizo software, have addressed some of these limitations. This software enables researchers to compare length, diameter, and the number of root tips between untreated and biostimulant-treated seeds, both in Petri dishes and pots.

In this study, the benefits resulting from the synergy of microbial strains present in the biostimulants used are evident and align with findings from previous research (Abdallah et al., 2021; Khan et al., 2018; Narendra Babu et al., 2015). The observed irregular dynamics in this study deserve further investigation, consistent with ongoing research on microbial biostimulants across various crops. The discrepancies between the populations in the Petri dishes might stem from variations in bacterial behavior. Notably, *Azospirilimum brasilense* was found to have a positive influence on the roots' architectural traits; however, these effects can vary based on the type of interaction established (Méndez-Gómez et al., 2021; Spaepen et al., 2014). For instance, the synthesis of indole-3-acetic acid, a phytohormone *Azospirillum* synthesizes in substantial amounts, can significantly enhance root growth when applied exogenously to tomato seeds (Mangmang et al., 2015). Thus, careful consideration in utilizing biostimulants is crucial, as the concentration of microbial strains plays a significant role in their effectiveness (Hwang et al., 2022).

In the incubator and pots with a sowing substrate, at two distinct phenological phases (13 and 15 on the BBCH scale), a greater number of microbial strains resulted in increased values for all analyzed root parameters. Therefore, the BS2 treatment resulted in statistically higher values than BS1, which exhibited statistically higher values than untreated plants. The additional presence of *Azotobacter chroococcum* in BS2 differentiates it from BS1. *A. chroococcum*, as highlighted in inoculation studies on corn subjected to water stress, is known to induce morphological improvements in roots, such as increased root length (Tyagi et al., 2023). Furthermore, the presence of *Priestia megaterium* in the *Crithmum maritimum* root system seems to have positive effects, especially when it colonizes the soil, benefiting other halophytes (Hwang et al., 2022).

5. Conclusion

Changes in root architecture are of great significance as they can directly affect the ability of plants to explore the soil and influence their capacity to uptake water and

nutrients. Biostimulants demonstrate resilience under a wide range of stress conditions, making them suitable as bacterial inoculants for agriculture, especially at low environmental impact or in marginal contexts where sea-fennel cultivation is viable. Considering the productive results observed for *Crithmum maritimum* in central Italy, as highlighted in Zenobi et al. (2022) and given the initial effect (BBCH 6 in an incubator and BBCH 13 in pots) of the population on the root parameters studied, *C. maritimum* L. is a crop that responds favorably to the four microbial inoculants used in this study. Indeed, the use of biostimulants demonstrated a stimulating effect on the root parameters analyzed in both the incubator and the greenhouse, increasing weight (approximately +75% on average), diameter (+25% on average), length (about +100% on average), and the number of tips (about +51% on average) compared to the control. These findings, observed in two controlled environments, suggest the potential use of biostimulants during nursery phases of production to promote the adaptation of alternative crops in cropping systems increasingly affected by climate change. The goal is to mitigate their adverse effects and foster plant growth.

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Contribution of authors
Roberto Orsini and Lucia Aquilanti worked on conceptualization; Biagio Di Tella performed data curation; Stefano Zenobi and Lucia Aquilanti conducted the investigation; Roberto Orsini and Vesna Milanovic organized resources; Marco Fiorentini and Luigi Ledda performed data analysis using software; Lucia Aquilanti and Roberto Orsini provided supervision; Stefano Zenobi, Andrea Marcelli, and Roberto Orsini wrote the original draft; and Roberto Orsini, Lucia Aquilanti, and Paola Deligios wrote the review and did the editing.

Conflict of interest
The authors declare no conflict of interest.

Data availability statement
Data are available upon reasonable request.

References

Abdallah I, Amer A, El-Hefny D (2021). Influence of herbicides under biofertilizer application on fennel (Foeniculum vulgare) yield and quality with special reference to herbicide residues. Bulletin of the National Research Centre 45 (1): 77. https://doi.org/10.1186/s42269-021-00534-w

Alemán A, Marín D, Taladrid D, Montero P, Carmen Gómez-Guillén M (2019). Encapsulation of antioxidant sea fennel (*Crithmum maritimum*) aqueous and ethanolic extracts in freeze-dried soy phosphatidylcholine liposomes. Food Research International 119: 665-674. https://doi.org/10.1016/j.foodres.2018.10.044

Alves-Silva JM, Guerra I, Gonçalves, MJ, Cavaleiro C, Cruz MT (2020). Chemical composition of *Crithmum maritimum* L. essential oil and hydrodistillation residual water by GC-MS and HPLC-DAD-MS/MS, and their biological activities. Industrial Crops and Products 149: 112329. https://doi.org/10.1016/j.indcrop.2020.112329

Amavizca E, Bashan Y, Ryu CM, Farag MA, Bebout BM et al. (2017). Enhanced performance of the microalga *Chlorella sorokiniana* remotely induced by the plant growth-promoting bacteria *Azospirillum brasilense* and *Bacillus pumilus*. Scientific Reports 7 (1): 41310. https://doi.org/10.1038/srep41310

Araujo FF, Bonifacio A, Bavaresco LG, Mendes LW, Araujo ASF (2021*). Bacillus subtilis* changes the root architecture of soybean grown on nutrient-poor substrate. Rhizosphere 18: 100348. https://doi.org/10.1016/j.rhisph.2021.100348

Atia A, Barhoumi Z, Mokded R, Abdelly C, Smaoui A (2011). Environmental eco-physiology and economical potential of the halophyte Crithmum maritimum L. (Apiaceae). Journal of Medicinal Plants Research 5 (16): 3564-3571.

Azeñas V, Janner I, Medrano H, Gulías J (2019). Evaluating the establishment performance of six native perennial Mediterranean species for use in extensive green roofs under water-limiting conditions. Urban Forestry & Urban Greening 41: 158-169: https://doi.org/10.1016/j.ufug.2019.04.002

Barbosa JZ, Roberto L de A, Hungria M, Corrêa RS, Magri E et al. (2022). Meta-analysis of maize responses to *Azospirillum brasilense* inoculation in Brazil: benefits and lessons to improve inoculation efficiency. Applied Soil Ecology 170: 104276. https://doi.org/10.1016/j.apsoil.2021.104276

Bertrand C, Gonzalez-Coloma A, Prigent-Combaret C (2021). Plant metabolomics to the benefit of crop protection and growth stimulation. Advances in Botanical Research 98: 107-132. https://doi.org/10.1016/bs.abr.2020.11.002

Buhmann A, Papenbrock J (2013). Biofiltering of aquaculture effluents by halophytic plants: basic principles, current uses and future perspectives. Environmental and Experimental Botany 92: 122-133. https://doi.org/10.1016/j.envexpbot.2012.07.005

Caruso G, De Pascale S, Cozzolino E, Giordano M, El-Nakhel C et al. (2019). Protein hydrolysate or plant extract-based biostimulants enhanced yield and quality performances of greenhouse perennial wall rocket grown in different seasons. Plants 8 (7): 208. https://doi.org/10.3390/plants8070208

Castillo JM, Mancilla-Leytón JM, Martins-Noguerol R, Moreira X, Moreno-Pérez AJ et al. (2022). Interactive effects between salinity and nutrient deficiency on biomass production and bio-active compounds accumulation in the halophyte *Crithmum maritimum*. Scientia Horticulturae 301: 111136. https://doi.org/10.1016/j.scienta.2022.111136

Chamkhi I, Sbabou L, Aurag J (2023). Improved growth and quality of saffron (*Crocus sativus* L.) in the field conditions through inoculation with selected native plant growth-promoting rhizobacteria (PGPR). Industrial Crops and Products 197: 116606. https://doi.org/10.1016/j.indcrop.2023.116606

Chen CY, Liu CM, Yeh HC, Wu HM, Li WJ et al. (2021). Flavonoids of *Crithmum maritimum*. Chemistry of Natural Compounds 57 (5): 917-920. https://doi.org/10.1007/s10600-021-03512-w

Colla G, Hoagland L, Ruzzi M, Cardarelli M, Bonini P et al. (2017). Biostimulant action of protein hydrolysates: unraveling their effects on plant physiology and microbiome. Frontiers in Plant Science 8. https://doi.org/10.3389/fpls.2017.02202

Corona BEL, Ocampo AG, Juárez DR, García JO, Fernández IM et al. (2023). Biostimulant effect of chitosan and phenolic extracts on the phenological development of the halophyte *Salicornia bigelovii* (Torr.). Journal of the Saudi Society of Agricultural Sciences 22 (8): 584-590. https://doi.org/10.1016/j.jssas.2023.08.001

Fleming TR, Fleming CC, Levy CCB, Repiso C, Hennequart F et al. (2019). Biostimulants enhance growth and drought tolerance in *Arabidopsis thaliana* and exhibit chemical priming action. Annals of Applied Biology 174 (2): 153-165. https://doi.org/10.1111/aab.12482

Gauri SS, Mandal SM, Pati BR (2012). Impact of *Azotobacter* exopolysaccharides on sustainable agriculture. Applied Microbiology and Biotechnology 95 (2): 331-338. https://doi.org/10.1007/s00253-012-4159-0

Generalić Mekinić I, Blažević I, Mudnić I, Burčul F, Grga M et al. (2016). Sea fennel (*Crithmum maritimum* L.): phytochemical profile, antioxidative, cholinesterase inhibitory and vasodilatory activity. Journal of Food Science and Technology 53 (7): 3104-3112. https://doi.org/10.1007/s13197-016-2283-z

Generalić Mekinić I, Šimat V, Ljubenkov I, Burčul F, Grga M et al. (2018). Influence of the vegetation period on sea fennel, *Crithmum maritimum* L. (Apiaceae), phenolic composition, antioxidant and anticholinesterase activities. Industrial Crops and Products 124: 947-953. https://doi.org/10.1016/j.indcrop.2018.08.080

Hamed KB, Messedi D, Ranieri A, Abdelly C (2008). Diversity in the response of two potential halophytes (*Batis maritima* and *Crithmum maritimum*) to salt stress. In: Abdelly C, Öztürk M, Ashraf M, Grignon C (editors). Biosaline Agriculture and High Salinity Tolerance. Basel, Switzerland: Birkhäuser Verlag. https://doi.org/10.1007/978-3-7643-8554-5_7

Hashem A, Tabassum B, Fathi AAE (2019). *Bacillus subtilis*: a plant-growth promoting rhizobacterium that also impacts biotic stress. Saudi Journal of Biological Sciences 26 (6): 1291-1297. https://doi.org/10.1016/j.sjbs.2019.05.004

Hulkko LSS, Chaturvedi T, Custódio L, Thomsen MH (2023). Harnessing the value of *Tripolium pannonicum* and *Crithmum maritimum* halophyte biomass through integrated green biorefinery. Marine Drugs 21 (7): 380. https://doi.org/10.3390/md21070380

Hwang HH, Chien PR, Huang FC, Yeh PH, Hung SHW et al. (2022). A plant endophytic bacterium *Priestia megaterium* strainBP-R2 isolated from the halophyte *Bolboschoenus planiculmis* enhances plant growth under salt and drought stresses. Microorganisms 10 (10): 2047. https://doi.org/10.3390/microorganisms10102047

Jallali I, Zaouali Y, Missaoui I, Smeoui A, Abdelly C et al. (2014). Variability of antioxidant and antibacterial effects of essential oils and acetonic extracts of two edible halophytes: *Crithmum maritimum* L. and *Inula crithmoïdes* L. Food Chemistry 145: 1031-1038. https://doi.org/10.1016/j.foodchem.2013.09.034

Jha B, Gontia I, Hartmann A (2012). The roots of the halophyte *Salicornia brachiata* are a source of new halotolerant diazotrophic bacteria with plant growth-promoting potential. Plant and Soil 356 (1-2): 265-277. https://doi.org/10.1007/s11104-011-0877-9

Khan N, Bano A, Zandi, P (2018). Effects of exogenously applied plant growth regulators in combination with PGPR on the physiology and root growth of chickpea (*Cicer arietinum*) and their role in drought tolerance. Journal of Plant Interactions 13 (1): 239-247. https://doi.org/10.1080/17429145.2018.1471527

Koli P, Bhardwaj NR, Mahawer SK (2019). Chapter 4 - agrochemicals: harmful and beneficial effects of climate changing scenarios. In: Choudhary KK, Kumar A, Singh AK (editors). Climate Change and Agricultural Ecosystems. Amsterdam, the Netherlands: Elsevier, pp. 49-70.

Mandal S, Ghorai M, Anand U, Roy D, Kant N et al. (2022). Cytokinins: a genetic target for increasing yield potential in the CRISPR era. Frontiers in Genetics 13. https://doi.org/10.3389/fgene.2022.883930

Mangmang JS, Deaker R, Rogers G (2015). Optimal plant growth-promoting concentration of *Azospirillum brasilense* inoculated to cucumber, lettuce and tomato seeds varies between bacterial strains. Israel Journal of Plant Sciences 62 (3): 145-152. https://doi.org/10.1080/07929978.2015.1039290

Marques DM, Magalhães PC, Marriel IE, Gomes Júnior CC, da Silva AB et al. (2023). Does *Azospirillum brasilense* mitigate water stress and reduce the use of nitrogen fertilizers in maize? South African Journal of Botany 156: 278-285. https://doi.org/10.1016/j.sajb.2023.03.041

Marques DM, Magalhães PC, Marriel IE, Gomes Júnior CC, da Silva AB et al. (2021). Gas exchange, root morphology and nutrients in maize plants inoculated with Azospirillum brasilense cultivated under two water conditions. Brazilian Archives of Biology and Technology 64. https://doi.org/10.1590/1678-4324-2021190580

Méndez-Gómez M, Castro-Mercado E, López-Bucio J, García-Pineda E (2021). *Azospirillum brasilense* Sp245 triggers cytokinin signaling in root tips and improves biomass accumulation in *Arabidopsis* through canonical cytokinin receptors. Physiology and Molecular Biology of Plants 27 (8): 1639-1649. https://doi.org/10.1007/s12298-021-01036-9

Meot-Duros L, Magné C (2008). Effect of salinity and chemical factors on seed germination in the halophyte *Crithmum maritimum* L. Plant and Soil 313 (1-2): 83-87. https://doi.org/10.1007/s11104-008-9681-6

Narendra Babu, A, Jogaiah S, Ito S, Kestur Nagaraj A, Tran LSP (2015). Improvement of growth, fruit weight and early blight disease protection of tomato plants by rhizosphere bacteria is correlated with their beneficial traits and induced biosynthesis of antioxidant peroxidase and polyphenol oxidase. Plant Science 231: 62-73. https://doi.org/10.1016/j.plantsci.2014.11.006

Nektarios PA, Nydrioti E, Kapsali T, Ntoulas N (2016). *Crithmum maritimum* growth in extensive green roof systems with different substrate type, depth and irrigation regime. Acta Horticulturae 1108: 303-308. https://doi.org/10.17660/ActaHortic.2016.1108.40

Pereira CG, Barreira L, da Rosa NN, Nogueira JMF, Marques C et al. (2017). Searching for new sources of innovative products for the food industry within halophyte aromatic plants: *in vitro* antioxidant activity and phenolic and mineral contents of infusions and decoctions of *Crithmum maritimum* L. Food and Chemical Toxicology 107: 581-589. https://doi.org/10.1016/j.fct.2017.04.018

Pereira CG, Moraes CB, Franco CH, Feltrin C, Grougnet R et al. (2021). In vitro anti-*Trypanosoma cruzi* activity of halophytes from southern Portugal reloaded: a special focus on sea fennel (*Crithmum maritimum* L.). Plants 10 (11): 2235. https://doi.org/10.3390/plants10112235

Piatti D, Angeloni S, Caprioli G, Maggi F, Ricciutelli M et al. (2022). Sea Fennel (*Crithmum maritimum* L.): a promising biosaline crop. Extraction, purification and chemical characterization of polar extracts. Biology and Life Sciences Forum 11(1): 61. https://doi.org/10.3390/IECPS2021-12033

Politeo O, Popović M, Veršić Bratinčević M, Kovačević K, Urlić B et al. (2023). Chemical profiling of sea fennel (*Crithmum maritimum* L., Apiaceae) essential oils and their isolation residual waste-waters. Plants 12 (1): 214. https://doi.org/10.3390/plants12010214

R Core Team (2019). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing: Vienna, Austria.

Renna M (2018). Reviewing the prospects of sea fennel (*Crithmum maritimum* L.) as emerging vegetable crop. Plants 7 (4): 92. https://doi.org/10.3390/plants7040092

Santini G, Biondi N, Rodolfi L, Tredici MR (2021). Plant biostimulants from cyanobacteria: an emerging strategy to improve yields and sustainability in agriculture. Plants 10 (4): 643. https://doi.org/10.3390/plants10040643

Souid A, Della Croce CM, Frassinetti S, Gabriele M, Pozzo L et al. (2021). Nutraceutical potential of leaf hydro-ethanolic extract of the edible halophyte *Crithmum maritimum* L. Molecules 26 (17): 5380. https://doi.org/10.3390/molecules26175380

Spaepen S, Bossuyt S, Engelen K, Marchal K, Vanderleyden, J (2014). Phenotypical and molecular responses of *Arabidopsis thaliana* roots as a result of inoculation with the auxin-producing bacterium *Azospirillum brasilense*. New Phytologist 201 (3): 850-861. https://doi.org/10.1111/nph.12590

Tyagi J, Mishra A, Kumari S, Singh S, Agarwal H et al. (2023). Deploying a microbial consortium of *Serendipita indica*, *Rhizophagus intraradices*, and *Azotobacter chroococcum* to boost drought tolerance in maize. Environmental and Experimental Botany 206*:* 105142. https://doi.org/10.1016/j.envexpbot.2022.105142

Wichard T, Bellenger JP, Morel FMM, Kraepiel AML (2009). Role of the siderophore azotobactin in the bacterial acquisition of nitrogenase metal cofactors. Environmental Science & Technology 43 (19): 7218-7224. https://doi.org/10.1021/es8037214

Yakhin OI, Lubyanov AA, Yakhin IA, Brown PH (2017). Biostimulants in plant science: a global perspective. Frontiers in Plant Science 7. https://doi.org/10.3389/fpls.2016.02049

Yao L, Liang D, Xia H, Pang Y, Xiao Q et al. (2023). Biostimulants promote the accumulation of carbohydrates and biosynthesis of anthocyanins in 'Yinhongli' plum. Frontiers in Plant Science 13. https://doi.org/10.3389/fpls.2022.1074965

Zenobi S, Fiorentini M, Ledda L, Deligios P, Aquilanti L et al. (2022). *Crithmum maritimum* L. Biomass production in Mediterranean environment. Agronomy 12 (4): 926. https://doi.org/10.3390/agronomy12040926

Zenobi S, Fiorentini M, Zitti S, Aquilanti L, Foligni R et al. (2021). *Crithmum maritimum* L.: first results on phenological development and biomass production in Mediterranean areas. Agronomy 11 (4): 773. https://doi.org/10.3390/agronomy11040773