Pol. J. Environ. Stud. Vol. 32, No. 6 (2023), 5129-5136
DOI: 10.15244/pjoes/169013 ONLINE PUBLICATION DATE: 2023-09-13

Original Research

Plant- and Animal-Derived Biostimulants for Overcoming Salinity Stress by Regulating the Physiological Parameters of Broccoli

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Received: 2 June 2023
Accepted: 29 June 2023

Abstract

Salinity depresses plant growth and final yield due to excessive toxic ions or their osmotic potential, resulting in physiological drought. This study focused on determining if plant- and animal-derived biostimulants (Aminolom Enzimatico®, İnori®, and Isabion®) improve plant growth, physiology, and stomata properties of the lower surface of leaves in broccoli under salinity. Broccoli seedlings were grown at the medium salinized with NaCl and control, and the biostimulants were applied to the soil (S), foliar (F), and S+F. Plant growth was inhibited by salinity, while the biostimulants enhanced the tolerance level to salinity. Stem diameter, plant fresh weight, and leaf area under salinity were improved by biostimulant applications, while S+F applications increased the fresh weight and leaf area under both saline and control. Electrolyte leakage precisely distinguished the differences between biostimulant applications. Among the biostimulants, İnori® effectively promoted morphological and physiological parameters, and there were also significant differences among the F, S, and F+S applications of the biostimulants. The F application was suitable for plant-derived biostimulants, while the S application of animal-derived biostimulant showed superiority. Therefore, plant-derived and animal-derived biostimulants should be applied on leaves and soil to reduce the hazardous effects of salinity in broccoli cultivation.

Keywords: *Brassica oleracea* var. *italica* L, NaCl, biostimulant, electrolyte leakage, stomata density

Introduction

Broccoli (*Brassica oleracea* var. *italica* L.), a cool-season vegetable, is grown in Türkiye under field and greenhouse conditions with irrigation to produce inflorescences. Due to its health-promoting properties through various bioactive compounds such as glucosinolates, phenols, vitamin C, and carotenoids, which may contribute to the reduction of cancer risk, the consumption of broccoli has continuously gained attention [1]. It is considered to be a moderately salt-sensitive crop by [2], but depends on genotypic factors [3]. They informed that salinity leads to limitations in plant growth and floret yield, while glucosinolates and phenolics content in florets were increased.

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Salinity is one of the most important environmental stresses caused by irrigation water or excessive ion content in the soil, which affects plant growth and development by destroying physiological processes. It causes ion imbalance due to high Na accumulation in plant tissues and physiological drought due to osmotic stresses with salt ions [4, 5]. To mitigate salinity stress in plants, various approaches such as the application of mycorrhizae, bacteria and fungi [6], seed priming [7], soil enrichment with organic matter and biofertilizers, foliar application of organic and inorganic substances or biostimulants have become popular in recent years [8-10].

Biostimulants, derived from various resources such as plants, animals, seaweeds, and fungi contain a variety of active compounds that stimulate plant growth and yield [11]. They are utilized as growth regulators that increase tolerance levels to abiotic stresses such as salinity [12], water deficit [13], drought [14], and cold [15]. The beneficial effects of biostimulants on salt-stressed plants have been demonstrated in numerous studies [16, 17]. They play a key role in improving photosynthesis, water uptake, and promotion of plant nutrients from the soil [18]. They also stimulate water availability, dry matter accumulation, and chlorophyll content despite a reduction in water content and membrane leakage under salinity [19, 20]. Biostimulants can be applied as foliar sprays or in the soil, and their efficacy depends on the type, timing, concentration, and content [21]. Therefore, this study focused on the selection of foliar spray and soil applications of plant- and animal-derived biostimulants (Isabion®, İnori®, and Aminolom Enzimatico®) on broccoli seedlings grown under salinity.

Experimental

A pot experiment was planned under laboratory conditions to determine the effects of biostimulants derived from animal and plant resources on circumventing salinity stress. Uniform broccoli seedlings from cultivar Belstar, purchased from a commercial seedling producer in Eskişehir-Türkiye, were transferred directly to plastic pots filled with a 3:1:1 mixture of peat, perlite, and vermiculite. The pots were 11 cm high, 10 cm in diameter at the top, and 9 cm at the bottom. They were watered to field capacity with a liquid N-P-K fertilizer (8-8-8) after transplantation, and the plants were placed in a growth chamber at 18ºC and relative humidity of 70% for 4 days to allow root growth. Then, the pots were divided into two main groups: saline and non-saline. Forty pots were attained for each group. Salinity was adjusted by a solution of 30 g NaCl per liter of distilled water with an electrical conductivity (EC) of 32.5 dS m^{-1}. Fifty milliliters of this solution were applied to each pot in the saline group, and the EC values in the growing medium were measured to be 7.2 dS m^{-1} using a portable moisture meter (HH2 Delta-T Devices, Cambridge, England). The pots were weighed to determine the degree of evaporation, and the amount of water lost was added at one-day intervals. The plants were grown on a cycle of approximately 22ºC/18ºC day/night temperatures for 16 h/8 h at 70% relative humidity.

Two commercial plant-derived biostimulants Aminolom Enzimatico® 24% (35% organic matter, 16% organic carbon, 2% organic nitrogen, and 24% free amino acids; glutamate 189 g L^{-1}, lysine 60 g L^{-1}, glycine 49 g L^{-1}, pH 4.5-6.5), İnori® (22% OM, 8.5% OC, 1% total nitrogen, 2.5% K_2O, with fulvic acid, free amino acids, and enzymes; pH 4.5-6.5) and the animal-based biostimulant Isabion® (62% OM, 30% OC, 10% ON, and 11% free amino acids; pH 6.5-7.5) were used in this study. Aminolom Enzimatico® and İnori® contained mainly vitamins, amino acids, betaines, and natural plant hormones. Isabion® was a biostimulant derived from animal collagen containing glycine, proline, hydroxyproline, and small peptides [12]. They were applied with irrigation water to soil (S), to the leaves (F) with manual hand sprayers, and soil + leaves (S + F) of broccoli seedlings twice 7 days after the salt treatment.

The experiment was planned as a two-factor factorial in a completely randomized design with four replicates. Biostimulants were prepared using a solution containing 2.5 ml L^{-1} water, as recommended by the manufacturer for vegetables, to which Tween-20 (0.1%) was added as an adhesive. For foliar application, a hand sprayer was used to evenly spray the plants. Soil application was done simultaneously with the foliar application, and 50 ml of the biostimulant solution were poured into each pot.

Thirty days after transplanting, stem diameter, chlorophyll content, and leaf temperature were measured using a portable digital caliper, a chlorophyll meter SPAD-502 (Konica Minolta Corporation, Osaka, Japan) as SPAD index, and an infrared thermometer (Trotec BP21). Immediately after the plants were harvested above the soil surface, the fresh weight of the plants was weighed, and the leaf area was determined on scanned leaves using the Image J program [22].

The second and third leaves from the top of each plant were used to determine relative water content (RWC) using the following equation.

$$RWC (\%) = [(W_f - W_d) / (W_t - W_d)] \times 100$$

In which,
W_f = the fresh weight of leaves,
W_d = the dry weight of leaves after drying at a constant temperature of 80 °C for 24 h, and
W_t = the turgid weight of leaves after samples were immersed in distilled water in a closed falcon tube for 24 h in the dark at 20ºC [23].

For electrolyte leakage, the third leaf from the top of each plant was cut and cleaned with distilled water. After the leaves were gently surface-dried with paper towels, four-six discs 5 mm in diameter were excised, weighed, and subsequently floated in 20 mL of distilled water in glass tubes, which were left for 24 h in an incubator at 20ºC in the dark. Electrical conductivity at the time point (EC_1, µS cm^{-1} g^{-1}) was read by a WTW 3.15i EC meter at 25ºC. Afterward, the tubes were incubated in a thermostatic water bath at 90ºC for 50 min to kill all cells, at which point the electrical conductivity was again read (EC_2, µS cm^{-1} g^{-1}) at 25ºC after they were cooled in an incubator. EL was calculated using the following equation [24]:

$$EL\ (\%) = (EC_1 / EC_2) \times 100$$

Stomata observations were made using the impression technique on the lower epidermis of leaves. The density of stomata was counted visually using a 40× objective lens and 10× eyepieces under a light microscope and photomicrographs were taken using a Zeiss Axiophot microscope [23]. The size of the stomata in the photograph was measured with the AxioVision 4.3 program using the stoma width and length in the following formula [25]:

$$\text{Stoma size } (\mu m^2) = [(\text{Stomata width} / 2) \times (\text{Stoma length} / 2)] \times \pi$$

Data were analyzed using the software JMP 13.0 version. Tukeys' HSD test at P<0.05 level was preferred to separate the statistical difference between control and biostimulant applications.

Results

Significant differences were found for all traits when biostimulants were applied, whereas the effect of salinity on stomata density was not significant (Table 1 and Table 2). A two-way interaction for the parameters studied was also found to be significant. In the presence of salinity, stem diameter, fresh weight, leaf area, stomata size, leaf temperature, and relative water content decreased, while dry matter, chlorophyll content, and electrolyte leakage increased. Plant fresh weight and leaf area decreased from 12.7 g and 241 cm^2, respectively, in the control group to 8.5 g and 160 cm^2 under salt stress. Electrolyte leakage in broccoli plants grown under salt stress increased from 8.3% to 19.7%. Broccoli plants had higher fresh weight, leaf area, and chlorophyll content when biostimulants were applied, with significant differences among biostimulant types and application methods. However, all parameters studied differed significantly in the

Table 1. Main effects of salinity and biostimulant applications on stem diameter, fresh weight, leaf area, dry matter, and chlorophyll content of broccoli.

Factor	Stem diameter (cm)	Fresh weight (g $plant^{-1}$)	Leaf area (cm^2)	Dry matter (%)	Chlorophyll content (SPAD)
Salinity (A)					
Control	4.34^{a}	12.7^{a}	241^{a}	10.5^{b}	61.3^{b}†
Salt stress	4.08^{b}	8.5^{b}	160^{b}	11.1^{a}	70.1^{a}
Treatments (B)					
No treatment	4.11^{d}	8.7^{e}	173^{d}	10.5^{bcd}	59.8^{c}
Aminolom Soil	4.45^{ab}	11.6^{abc}	217^{abc}	11.4^{abc}	68.2^{a}
Aminolom Foliar	4.11^{d}	10.6^{cd}	198^{b-d}	10.5^{bcd}	69.9^{a}
Aminolom S + F	4.55^{a}	12.5^{a}	231^{a}	11.4^{abc}	67.9^{ab}
İnori Soil	4.23^{bc}	12.4^{ab}	226^{ab}	11.8^{ab}	69.4^{a}
İnori Foliar	4.41^{ab}	11.1^{abc}	207^{abc}	9.5^{d}	62.5^{bc}
İnori S + F	4.39^{ab}	12.3^{ab}	232^{a}	10.0^{cd}	66.5^{ab}
İsabion Soil	4.13^{cd}	11.0^{bc}	216^{abc}	12.3^{a}	68.7^{a}
İsabion Foliar	3.78^{e}	9.1^{de}	168^{d}	10.7^{bcd}	69.6^{a}
İsabion S + F	4.01^{d}	10.4^{cd}	193^{cd}	10.6^{bcd}	66.6^{ab}
A	**	**	**	**	**
B	**	**	**	**	**
A × B	**	**	**	**	**

†Different superscript letters within each column refer to significance levels at P<0.05. **: significant at P<0.01. S: soil and F: foliar application.

Table 2. Main effects of salinity and biostimulant applications on stomata density, stomata size, leaf temperature, relative water content, and electrolyte leakage of broccoli.

Factor	Stomata density (number mm^{-2})	Stomata size (μm^2)	Leaf temperature (°C)	Relative water content (%)	Electrolyte leakage (%)
Salinity (A)					
Control	321	244^{a}	22.4^{a}	81.2^{a}	8.3^{b}†
Salt stress	322	227^{b}	22.0^{b}	69.3^{b}	19.7^{a}
Biostimulant (B)					
No treatment	287^{b}	251^{ab}	21.2^{de}	73.9^{de}	16.4^{b}
Aminolom Soil	358^{a}	216de	21.0^{e}	73.2^{de}	18.1^{a}
Aminolom Foliar	317^{ab}	255^{a}	21.0^{e}	76.2^{abc}	11.0^{ef}
Aminolom S + F	326^{ab}	250^{abc}	21.3^{de}	74.8^{cd}	12.5^{d}
İnori Soil	318^{ab}	228^{b-e}	22.2^{c}	77.1^{ab}	12.6^{d}
İnori Foliar	320^{ab}	237^{a-d}	21.3^{de}	77.8^{a}	10.5^{f}
İnori S + F	322^{ab}	235^{a-d}	22.0^{cd}	76.4^{abc}	11.1^{ef}
İsabion Soil	370^{a}	203^{e}	25.4^{a}	72.8^{e}	15.7^{bc}
İsabion Foliar	333^{ab}	225^{cde}	24.3^{b}	77.0^{ab}	12.1^{de}
İsabion S + F	333^{ab}	225^{cde}	24.1^{b}	75.8^{bc}	15.0^{c}
A	*ns*	**	**	**	**
B	**	**	**	**	**
A × B	**	**	**	**	**

†Different superscript letters within each column refer to significance levels at P<0.05. **: significant at P<0.01, ns: nonsignificant. S: soil and F: foliar application.

interaction of salinity and biostimulant applications (Fig. 1 and Fig. 2).

All three biostimulants promoted fresh weight of broccoli plants under both control and salinity conditions, but the beneficial effect of Isabion® was less than that of the plant-based biostimulants (Fig. 1a). On the other hand, S application produced higher fresh weight than F application. S and S + F applications of İnori® gave the highest fresh weight in salt-stressed broccoli plants. No significant effect of Isabion® was observed in plants grown under salt stress. However, S application of Isabion® caused a significant increase in dry matter under salt stress (Fig. 1b). Biostimulants had no effect on stem diameter of broccoli plants under salt stress, but S and S+F applications of Aminolom Enzimatico® promoted it significantly under non-saline conditions (Fig. 1c). Leaf temperature was affected by salinity and biostimulant applications, reaching the highest value in S application of Isabion® under control (Fig. 1d). However, the differences between applications did not appear to be meaningful in clarifying the benefits of biostimulants. A greater leaf area was measured in plants grown under control than in plants grown under salt stress (Fig. 1e). All biostimulants significantly increased leaf area, except for the F application of Isabion®. Under salt stress, S+F and S applications of İnori® increased it by 69% and 41%, respectively. Chlorophyll content was higher in salt-stressed broccoli than in control plants, and both plant- and animal-based biostimulants promoted it significantly. In broccoli plants under salt stress, chlorophyll content was improved from 65.7 SPAD to 76.7 SPAD by the F application of Aminolom Enzimatico® (Fig. 1f).

The size of stomata was smaller in plants grown under salt stress than in control plants, and the smallest stomata were obtained in the S application of Aminolom Enzimatico® (Fig. 2a). Similarly, high stomata frequency was achieved by biostimulant applications. Soil application of Aminolom Enzimatico® resulted in the highest stomata density with 391 numbers per mm^2 (Fig. 2b). An obvious difference was observed between the RWC of control and salt-stressed broccoli plants. The effect of biostimulants also depended on salt stress. The highest RWC was obtained with the F application of Aminolom Enzimatico® in the control, while the F application of İnori® produced in the salt-stressed plants (Fig. 2c). A similar trend was observed in EL, which decreased under salinity. Foliar application of biostimulants effectively decreased EL and the minimum EL was achieved by F application of Aminolom Enzimatico® (Fig. 2d). However, all types of

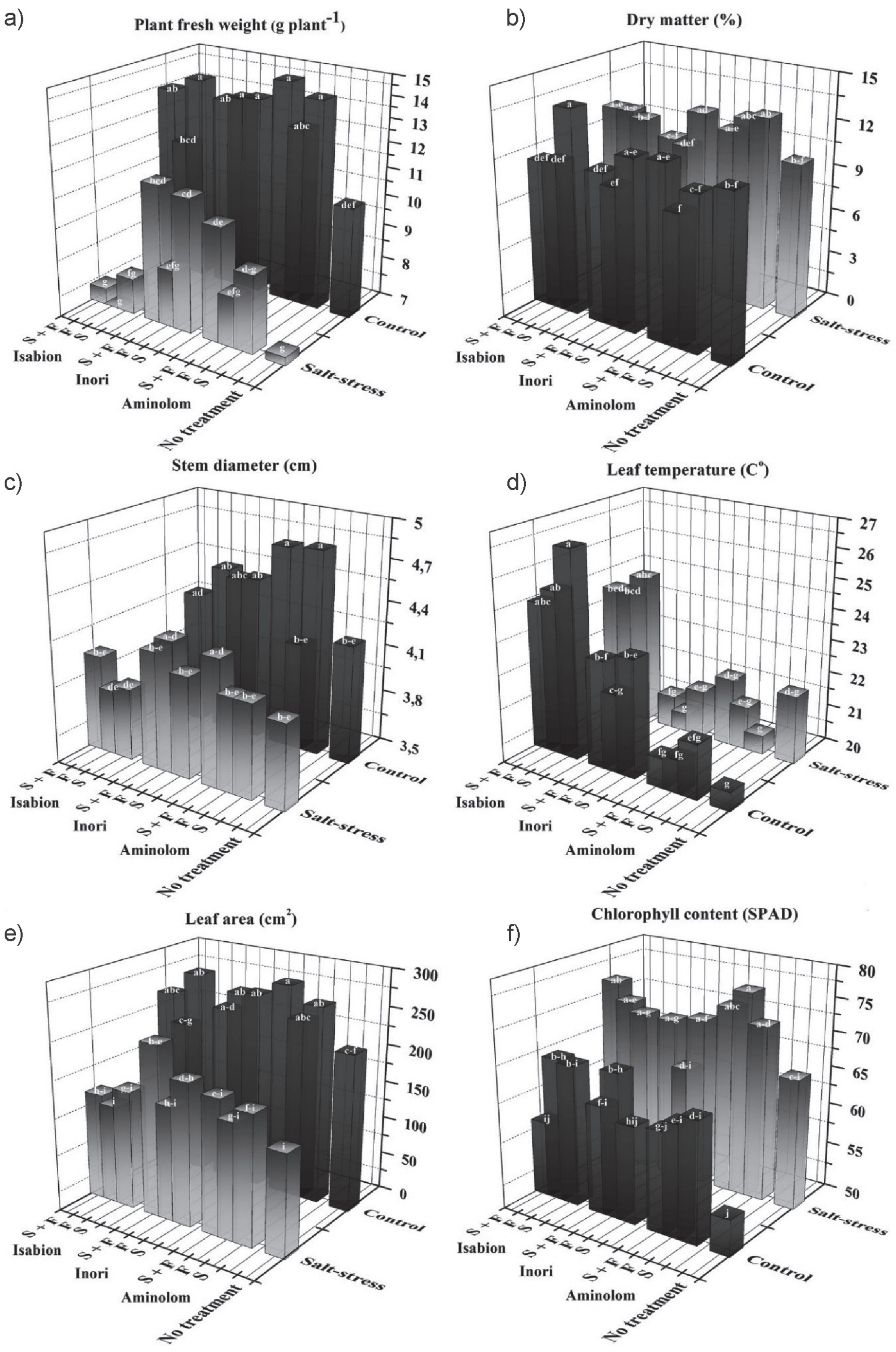

Fig. 1. The interaction of salinity and biostimulant applications on plant fresh weight a), dry matter b), stem diameter c), leaf temperature d), leaf area e), and chlorophyll content f). S: soil application, F: foliar application. Letters on each bar denote the significance level at p<0.05.

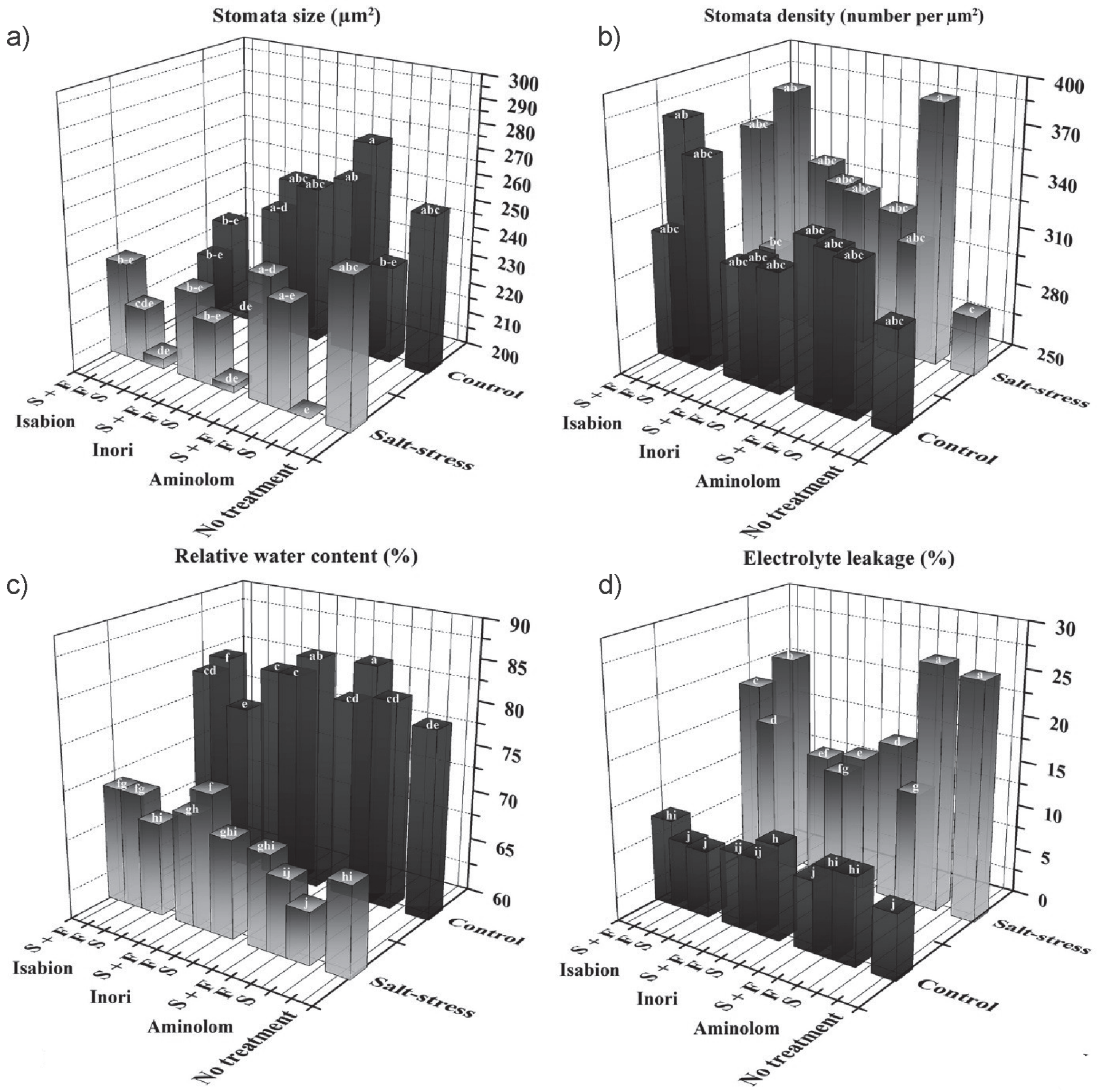

Fig. 2. The interaction of salinity and biostimulant applications on stomata size a), stomata density b), relative water content c), and electrolyte leakage d). S: soil application, F: foliar application. Letters on each bar denote the significance level at $p<0.05$.

application of İnori® showed lower EL than the other biostimulants.

Discussion

There are a variety of environmental stresses caused by climatic conditions such as drought, frost, and high temperatures, or by soil properties such as salinity, heavy metals, and extreme pH levels that result in growth limitation in crops. Soil salinity damages the ion balance in plant tissues or causes physiological drought due to water uptake from excessive salt ions. Recently, plant- or animal-derived biostimulants, some of which have been fortified with plant nutrients and phytohormones, have been proposed to stimulate plant growth in salt-affected areas by applying them with foliar or irrigation water. In the present study, the application of one biostimulant of animal origin (Isabion®) and two biostimulants of plant origin (Aminolom Enzimatico® and İnori®) via soil, foliage, soil, and foliage was investigated in broccoli plants grown under salt stress.

Growth of broccoli plants was strongly affected by soil salinity. Salinity reduced the fresh weight of broccoli by 29% and leaf area by 36%. At the same time, the application of all biostimulants contributed to the growth of salt-stressed broccoli plants. Aminolom Enzimatico® and İnori® induced leaf expansion and stem elongation more than Isabion®. These results support the fact that there are significant differences between biostimulants whose efficiency depends on the ingredients. Similar results were also reported by [12] and [26] in tomato, [27] in maize, and [28] in *Puccinellia distans*, which found that the adverse effects of salinity were mitigated by the application of biostimulants. The reasons for the improvement in morphological characteristics of salt-stressed broccoli plants are mainly due to the regulation of physiological parameters such as leaf temperature, dry matter, electrolyte loss, stomata size and density. Under salt stress, leaf temperatures and leaf tissue dry matter increased, while biostimulants slightly decreased them. Similarly, [29] demonstrated higher leaf temperatures in lettuce grown under increasing salinity, while it declined in plants sprayed with a plant-derived biostimulant. In stressed plants, an increase in dry matter was generally observed, and the biostimulants enhanced water content in tissues. This result confirms the findings of [21], who described a small accumulation of dry matter in spinach leaves when biostimulants were applied. However, smaller stomata and higher densities of stomata were found in rocket and lettuce under salinity [23, 29], which is comparable to the results of the present study on broccoli. Biostimulants helped to attenuate the deleterious effects of salinity, resulting in increased stomata size and frequency in the lower epidermis of broccoli leaves. Surprisingly, plants with all biostimulants applied to the soil formed larger and more frequent stomata than the other applications. Unfortunately, there were no research results to compare our stomata observations altered by biostimulant application. The salt-stressed plants were found to have a lower RWC than the control, which was not a surprising result based on previous reports by [30, 31, 28, and 29]. In the present study, foliar spraying with İnori® resulted in a significant improvement in RWC. Measurement of cell membrane stability and electrolyte leakage can be successfully used to discriminate between salt-tolerant and salt-sensitive plants [32]. Electrolyte leakage is a valuable indicator of salt-tolerant and -susceptible plants [32, 33]. The superiority of biostimulant application was most evident at EL in broccoli plants exposed to salinity. Although no significant changes in EL were observed in the control plants when biostimulants were applied, they showed a significant reduction in the plants exposed to salinity. This study showed that EL can even classify biostimulant application methods and resources. Moreover, there were significant differences among biostimulants, and plant-derived biostimulants were found to be the most effective in alleviating salt stress. [28] reported significant differences between two biostimulants, and an amino acid-derived biostimulant mitigated the unfavorable effects of salinity. In this study, differences between soil and foliar applications were clearly noticed. İnori® was the most effective biostimulant for balancing EL under salinity, but foliar applications of Aminolom Enzimatico® and Isabion® showed lower EL results than the others.

In conclusion, given the negative effects of salinity on plant growth and physiology, both animal- and plant-derived biostimulants may be useful for restoring plant production in salinity-stressed soils. Foliar application of plant-derived biostimulants can mitigate plant growth inhibition by regulating physiological properties under salinity. Soil application of the animal-derived biostimulant Isabion® was more beneficial than foliar application under saline or non-saline conditions. This could be due to the selectivity of plant organs for biostimulants with different resources, which is probably due to the different response mechanisms of biostimulants to salt stress. The results indicate that soil application of the animal-based biostimulant Isabion® and foliar application of the plant-based biostimulants Aminolom Enzimatico® and İnori® are recommended for salt-stressed broccoli plants.

Acknowledgments

The author would like to thank Prof. Dr. Süleyman AVCI for permission to observe the stomata with a microscope, and Dr. Nurgül ERGIN, Dr. Engin Gökhan KULAN and Pınar HARMANCI for their kind help.

Conflict of Interest

The authors declare no conflict of interest.

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