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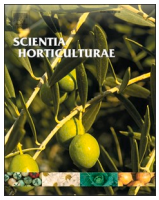

Research Paper

Biostimulant-induced mitigation of cold and drought stresses in zucchini plants

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ABSTRACT

Currently, water and cold stresses are among the primary adverse factors affecting global crop production. Biostimulants are increasingly recognized as valuable tools for enhancing plant tolerance to abiotic stresses. F4.3S is a novel biostimulant that contains compounds such as allantoin, ascorbate, salicylic acid, amino acids such as glutamate, proline, and glycine, and sources of selenium, molybdenum, and cobalt. The objective of this study was to assess the tolerance of zucchini cv. Dynaic plants supplied with F4.3S to water and cold stresses and to identify the potential action mechanisms. For this purpose, an experiment was set up in pots with plants to which the biostimulant was applied before and after subjecting the plants to stress conditions, and parameters of biomass, stress, photosynthesis, and ethylene response were evaluated. The results showed that plants supplied with F4.3S presented a better tolerance to both stresses, which was reflected in higher biomass. The potential action mechanisms could be the stimulation of photosynthetic efficiency, preventing excessive stomatal closure, maintaining a high rate of net photosynthesis, and reducing reactive oxygen species generation. Additionally, increased accumulation of protective anti-stress compounds such as proline and carotenoids, along with reduced ethylene synthesis, likely contributed to the plants' enhanced recovery post-stress. In conclusion, the F4.3S biostimulant emerges as a promising agent for augmenting plant tolerance to abiotic stresses, which is crucial for sustainable agricultural practices.

1. Introduction

Currently, the main abiotic factors worldwide are water stress, thermal stress (either due to low or high temperatures), and soil salinity (Ahmed et al., 2022; Alotaibi, 2023). The appearance of these unfavorable conditions for plant growth causes changes in morphological, physiological, and biochemical responses, with a consequent reduction in growth, yield, biomass, and quality. Hence, these stresses represent a serious problem for commercial horticulture, as they lead to a loss of productivity, particularly in the Mediterranean region (Pour-Aboughadareh et al., 2019; Song et al., 2019; Novák et al., 2021).

The first symptoms of plant stress are rapid inhibition of shoot growth (and to a lesser extent, root growth), followed by partial or complete stomatal closure, causing a reduction in transpiration and CO_2 absorption necessary for photosynthesis (Ilyas et al., 2021; Muhammad et al., 2021). The damage caused by these types of abiotic stresses is mainly due to two reasons: the formation of reactive oxygen species (ROS) and alteration of the plant's water relations. ROS have detrimental effects on biological structures, such as DNA damage and oxidation of amino acids, proteins, and lipids (Zhang et al., 2022). Therefore, the degree of resistance to these stresses is based on the capacity of plants to avoid or reduce the presence of these physiological processes (Hasanuzzaman et al., 2021; Ilyas et al., 2021). Furthermore, the induction of ethylene synthesis under stress generally has a negative consequence for the growth of most plants under unfavorable conditions because ethylene is responsible for massive ROS generation and the appearance of chlorosis, senescence, and cell death (Fatma et al., 2022). Different studies have suggested that the reduction of ethylene leads to the synthesis of antioxidant and osmoprotective compounds under stress conditions, such as glutathione (GSH) and glycine-betaine (Thao et al., 2015).

Different compounds have been used to reduce ethylene synthesis and therefore improve the resistance of plants to abiotic stress conditions (Fatma et al., 2022). Currently, the use of these types of

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compounds with physiological action in plants is being intensively developed through the so-called biostimulants. These products are receiving significant interest from researchers, industrial companies, and farmers as an effective and useful tool to improve crop productivity (D'Addabbo et al., 2019). Biostimulants are defined as products that are applied externally to plants in low concentrations to stimulate plant growth and development, stress tolerance, defense against pathogens, and reproductive development (Dalal et al., 2019; du Jardin, 2015). Thus, biostimulants induce defense responses such as the activation of the antioxidant system or the synthesis of osmoprotective compounds in plants. The marked effects of biostimulants have been observed in the control of drought, heat, salinity, cold, frost, oxidative, mechanical, and chemical stresses (du Jardin, 2015; Zulfiqar and Ashraf, 2021). Besides, under different abiotic stress conditions, it was confirmed that the application of different biostimulants can modulate leaf gas exchanges and water use efficiency (WUE) and, in general, provide greater photosynthetic efficiency by increasing the total chlorophyll (Chl) index and boost antioxidant defenses (Van Oosten et al., 2017; Bulgari et al., 2019; Rajabi Hamedani et al., 2020)

A wide variety of compounds are included in biostimulant formulations. Thus, the application of compounds such as allantoin, ascorbate, or salicylic acid was shown to enhance stress tolerance by influencing antioxidant capacity or hormonal regulation (Farhangi-Abriz and Ghassemi-Golezani, 2018; Bulgari et al., 2019; Deng et al., 2020; Kaur et al., 2021). Amino acids are among the other commonly added components to biostimulants. These provide an additional nitrogen source (glutamate) for growth or may serve osmoprotective functions (proline) or contribute to the synthesis of GSH (cysteine), which are crucial for stress tolerance (Barrameda-Medina et al., 2014; Carillo et al., 2019; Navarro-León et al., 2022). Alternatively, the addition of certain compounds that contain mineral elements, such as Se, Mo, and Co, has also been demonstrated to be useful in improving stress tolerance by enhancing antioxidant metabolism or promoting nitrogen assimilation (Campobenedetto et al., 2020; Medrano-Macías and Narvaéz-Ortiz, 2022; Elshamly, 2023). A proper combination of these compounds can increase the stress protection of crops (du Jardin, 2015).

Information is lacking about the specific effects of biostimulants on the biochemical and physiological mechanisms in the resistance of plants subjected to abiotic stress and about the combined effect of multiple bioactive compounds in biostimulants. The biostimulant assayed in this study (F4.3S) contains compounds such as allantoin, ascorbate, salicylic acid, amino acids such as glutamate, proline, and glycine, and sources of Se, Mo, and Co. Therefore, in the present study, the tolerance to water and cold stresses was assessed in zucchini cv. Dynaic plants to which an anti-stress biostimulant was applied. The present study aimed to elucidate its effectiveness and action mechanisms, including a potential reduction in ethylene synthesis. For this purpose, parameters of plant biomass, stress indicators, photosynthesis performance, and ethylene concentration and synthesis were evaluated.

2. Material and methods

2.1. Plant material and growing conditions

Zucchini plants (*Cucurbita pepo* L. cv. Dynaic F1) were used for the experiment. The seeds of these plants germinated and grew for 45 days in a tray with cells (cell size, 3 cm x 3 cm x 10 cm) at Saliplant S.L. (Carchuna, Granada). Subsequently, the seedlings were transferred to a culture chamber of the Department of Plant Physiology of the University of Granada under controlled conditions: relative humidity 60–80%, temperature 25 °C/15 °C (day/night), and 16 h/8 h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 $\mu mol^{-2}s^{-1}$ (measured with an SB quantum 190 sensor, LI – COR Inc., Lincoln, NE, USA).

Under these conditions, the plants grew in individual pots (13 cm upper diameter, 10 cm lower diameter, 12.5 cm high, and a volume of 2 L) filled with a vermiculite:perlite (3:2 ratio) mixture and arranged in trays (8 pots per tray). Fertilization consisted of a complete Hoglandtype nutrient solution, with small modifications for zucchini cultivation, composed of 4 mM KNO_3, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM NaH_2PO_4, 1 mM KH_2PO_4, 125 μM Fe-EDDHA, 50 μM H_3BO_3, 2 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, and 0.1 μM Na_2MoO_4, with a pH of 5.8. This nutrient solution (1.5 L) was applied to the trays every 3 days.

2.2. Description of treatments and experimental design

In this experiment, the effect of the application of the anti-stress biostimulant F4.3S provided by Atlántica Agrícola S.L. company was analyzed. The biostimulant contains 6.5% w/w free amino acids (2.5 % glutamic acid, 2 % glycine, and 2 % proline), 0.5% w/w ammonium heptamolybdate, 0.05% w/w EDTA-Co, 0.016% w/w sodium selenate, and 100 ppm allantoin, 100 ppm sodium salicylate, 50 ppm ascorbic acid, 35 ppm B3 vitamin, 5 ppm B6 vitamin, 0.05 ppm B2 vitamin. The treatments began 25 days after germination, initially applying the anti-stress product F4.3S to the leaves using a sprayer at a dose of 5 ml L^{-1}. Then, after 24 h, these plants were subjected to water stress and cold stress treatments. Once the plants showed clear symptoms of stress (chlorosis and wilting), the stresses were eliminated (after nine days in the case of cold stress and after seventeen days for water stress), and the plants were transferred to control conditions. At this time, the anti-stress product F4.3S was applied again foliarly at a dose of 5 ml L^{-1} to cold and drought-stressed plants. Finally, sampling of the plant material was carried out 7 days after the application of the F4.3S product. The study was structured as a completely randomized block design, involving eight plants for each treatment. Fig. S1 shows a timeline with the dates of treatment application and samplings. The plants were individually potted and placed in a random arrangement within the growth chamber. The different treatments applied to the plants are described in Table 1.

2.3. Plant sampling

Sampling of plant material was performed 7 days after the last application of the anti-stress product F4.3S (02/12/2022 for cold stress and 02/20/2023 for drought stress). Immediately after collection, all samples from each treatment group were prepared for further analysis. The plants were first cleaned, dried with filter paper, and weighed to measure their fresh weight (FW). Half of the fresh samples were frozen at −40 °C and used for the analysis of the concentration of photosynthetic pigments, malondialdehyde (MDA), ROS ($O_2^{\cdot-}$ and H_2O_2), proline, ethylene precursor 1-amino-cyclopropane carboxylic acid (ACC), and ACC oxidase activity. The remaining samples, after oven drying, were used for determining dry weight (DW).

2.4. Plant analysis

2.4.1. Relative water content

Leaf relative water content (RWC) was analyzed in the leaves of the plants at the end of the experiment. Leaves were cut and their FW was immediately recorded. Subsequently, they were placed in petri dishes, covered with distilled water, and kept for 4 h at room temperature and under constant light. They were then weighed and their turgid weight (TW) was obtained. These leaves were dried for 24 h at 80 °C in a forced

Table 1
Description of the treatments applied in the experiments to the zucchini plants.

Treatments	Description
Control	Cultivation under normal growing conditions
Cold	4 °C for 5 h a day
Cold + F4.3S	4 °C for 5 h a day + application of F4.3S one day before the start of stress and at the end of stress
Drought	Water stress at 0 % field capacity
Drought + F4.3S	Water Stress at 0 % field capacity + application of F4.3S one day before the start of stress and at the end of stress

air oven, thus obtaining the DW. The RWC was calculated according to the following formula (Barrs and Weatherley, 1962):

$$RWC\ (\%) = [(FW - - DW) / (TW - - DW)] \times 100$$

2.4.2. Electrolyte leakage

The stability of cell membranes was determined by performing electrolyte leakage (EL) test (Soloklui et al., 2012). To do this, 0.3 g of fresh plant material was weighed, cut into pieces, washed slightly with deionized water, and placed in a test tube. Then, 30 mL of deionized water was added and the tubes were shaken in a vortex for 1 min. Using a conductivity meter (Cond 8; XS Instruments, Italy) the initial conductivity (EC1) was measured. Subsequently, the tubes were incubated in a water bath at a temperature of 100 °C for 20 min to extract the released electrolytes, and they were allowed to cool to room temperature. Subsequently, the final conductivity (EC2) was measured. The percentage of EL was calculated using the following formula: (EC1/EC2) x 100.

2.4.3. Concentration of photosynthetic pigments

The concentration of photosynthetic pigments was analyzed by the method of Wellburn (1994) with certain modifications. 0.1 g of plant material was ground in 1 ml of methanol. Subsequently, it was centrifuged for 5 min at 5000 *g*. The absorbance was measured at 3 different wavelengths: 666 nm, 653 nm, and 470 nm, and the following calculations were made based on the following equations:

- Chl *a* = 15.65 x A666 nm–7.34 x A653 nm
- Chl *b* = 27.05 x A653 nm–11.21 x A666 nm
- Carotenoids = (1000 x A470 nm–2.86 x Chl *a* – 129.2 X Chl *b*) / 221

Chl *a* and Chl *b* concentrations were expressed as mg g^{-1} FW and carotenoids concentration was expressed as μg g^{-1} FW.

2.4.4. CHL a fluorescence analysis

Prior to the measurements, plants underwent a 30-minute dark adaptation phase. Measurements of Chl *a* fluorescence kinetics were conducted using the Handy PEA Chlorophyll Fluorimeter by Hansatech Ltd. This involved using a specific leaf clip on individual leaves and inducing OJIP phases with red light (650 nm) at an intensity of 3000 μmol photons $m^{-2}s^{-1}$. The OJIP fluorescence stages were examined using the JIP test on fully developed leaves from the middle section of six plants for each treatment. The JIP test provided various parameters to assess energy flows and photosynthetic efficiency: initial fluorescence (Fo), maximum fluorescence (Fm), variable fluorescence (Fv= Fm-Fo), maximum quantum product of primary photochemistry (Fv/Fm), electron entrapment potential (ψ_{Eo}), performance index (PIABS), and proportion of active reaction centers (RC) (RC/ABS) (Strasser et al., 2000).

2.4.5. Gas exchange measurements

Gas exchange measurements were conducted using a LICOR 6800 Portable Photosynthesis System IRGA. Measurements were taken on intermediate leaves set in cuvettes under ideal growth conditions. The instrument, after a 30-minute warm-up and calibration, measured net photosynthesis rate (*A*), transpiration rate (*E*), and stomatal resistance (r) under specific conditions: 400 μmol mol^{-1} CO_2, 60 % humidity, 500 μmol m^2 s^{-1} PAR, and 30 °C leaf temperature. Data were logged on the LICOR system and analyzed using Photosyn Assistant software, with Water Use Efficiency (WUE) calculated as the ratio of A to E. The units for gas exchange parameters were as follows: *A* (μmol m^{-2} s^{-1}), r (s cm^{-1}), *E* (mmol m^{-2} s^{-1}), and WUE (μmol $mmol^{-1}$).

2.4.6. Determination of the concentration of oxidative indicators (MDA, H_2O_2, and O_2^-) by spectrophotometry

To determine MDA concentration, the procedure described by Fu and Huang (2001) was followed. MDA concentration was expressed as μM g^{-1} FW. The H_2O_2 levels were determined colorimetrically according to Junglee et al. (2014). The O_2^- concentration was measured following the method described by Barrameda-Medina et al. (2014) based on the reaction with hydroxylamine, the formation of NO_2^- and its subsequent measurement by spectrophotometry. H_2O_2 and O_2^-concentrations were expressed as μg g^{-1} FW.

2.4.7. Determination of proline concentration

To determine the free proline concentration of the leaves, they were homogenized in 5 ml of 96 % ethanol. The insoluble fraction of the extract was washed with 5 ml of 70 % ethanol. The extract was centrifuged at 3500 *g* for 10 min and the supernatant was kept at 4 °C for proline determination according to the method described by Irigoyen et al. (1992). Proline concentration was expressed as μg g^{-1} FW.

2.4.8. Determination of ACC concentration

ACC concentration was analyzed using an U-HPLC-MS system as described by Navarro-Morillo et al. (2023) and expressed as ng g^{-1} DW.

2.4.9. Determination of ACC oxidase activity

A total of 0.1 g of leaf was homogenized with 1 ml of 0.05 M Tris–HCl buffer (pH 8.0) and centrifuged at 5000 *g* for 30 min at 4 °C. The supernatant was used for the ACC oxidase activity assay (Van de Poel et al., 2014). An amount of supernatant was added to an activity buffer containing 50 mM MOPS, 5 mM ascorbic acid, 20 mM sodium bicarbonate, 10 % glycerol, 0.1 mM DTT, and ACC (50 μg). The reaction was incubated in 4 mL vials for 60 min at 30 °C while shaking. A 1 ml sample was taken and the ACC remaining after the reaction was analyzed as described in Section 2.4.8. ACC oxidase activity was expressed as nmol mg $protein^{-1}$ min^{-1}.

2.5. Statistical analysis

Each analysis was conducted in triplicate and the data were statistically analyzed using an ANOVA test with a 95 % confidence level. Differences among treatment means were assessed using Fisher's least significant difference (LSD) test at a 95 % probability level. Significance was denoted as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; N.S. denoting not significant.

3. Results and discussion

3.1. Plant biomass, RWC, and EL

The parameters that most reliably indicate the presence of abiotic stresses are those associated with plant growth (He et al., 2018). To assess the impact of the biostimulant against cold stress and water deficit, we evaluated the production of fresh and dry biomass in the shoot. Generally, these parameters are reliable indicators of plant growth under varying conditions and, consequently, reflect their adaptive capacity to unfavorable environmental factors (Brown and Saa, 2015; Rakkammal et al., 2023).

As depicted in Figs. 1 and 2, both cold and drought stresses resulted in a decrease in plant growth, with the affected plants exhibiting the lowest shoot biomass among all treatments. Relative to the control, the following reductions in biomass were observed: a 61 % decrease in FW under cold stress, a 55 % decrease in DW under cold stress, a 77 % decrease in FW under drought stress, and a 62 % decrease in DW under drought stress. Drought stress caused a greater reduction in plant growth than cold stress (Figs. 1 and 2). On the other hand, we verified the positive detoxification effect of the F4.3S product on the stimulation of biomass production once the stress had ended and 7 days after its foliar application. Under both stress conditions, the application of F4.3S mitigated the reduction in both fresh and dry biomass compared with the control plants. The reductions in biomass due to cold stress were 49 % for FW and 40 % for DW. For drought stress, the decreases were 56 %

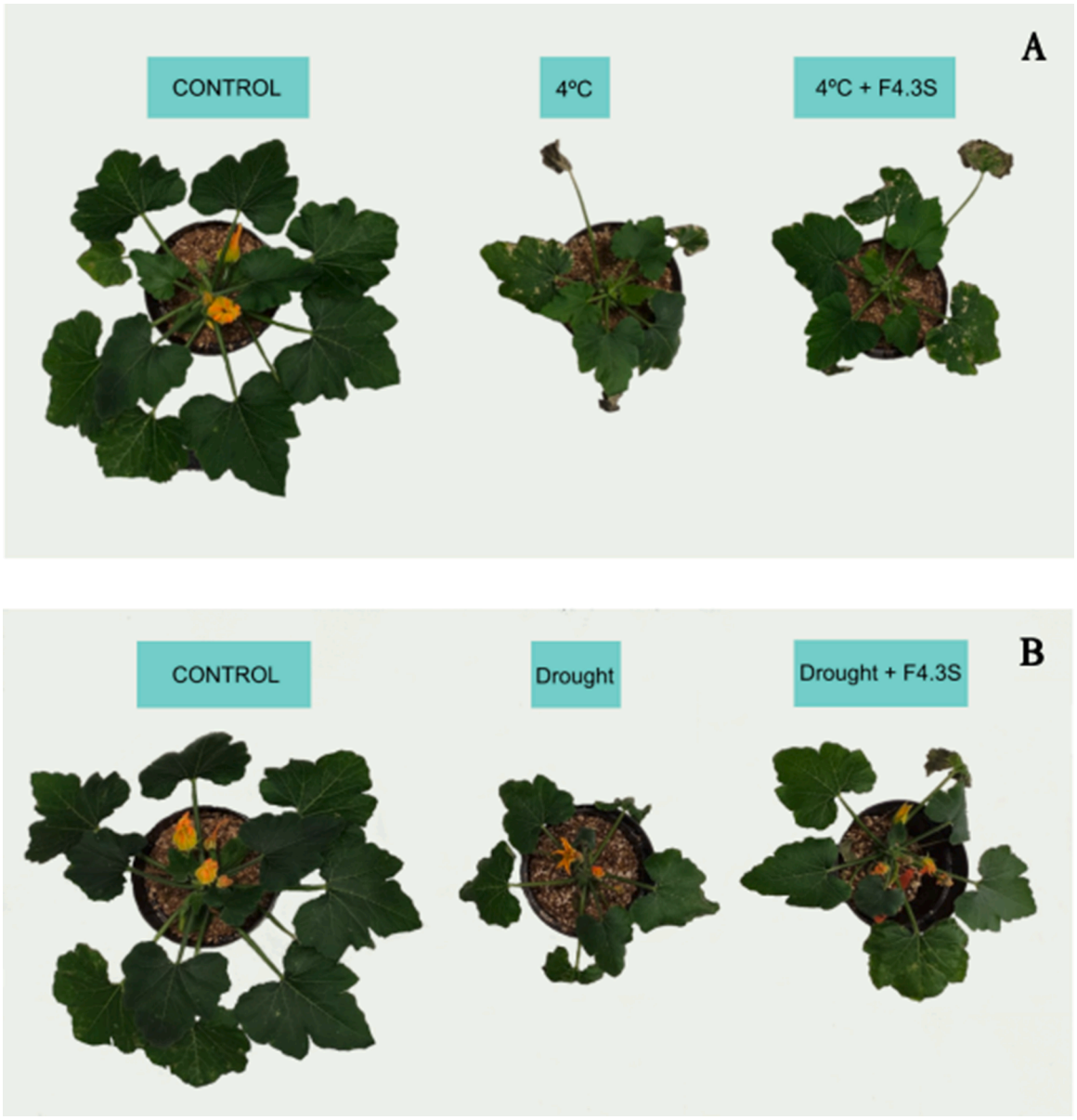

Fig. 1. Appearance of the zucchini plants subjected to cold stress and cold stress + F4.3S (A) and to drought and drought + F4.3S (B) at the sampling time.

for FW and 21 % for DW of the shoot (Figs. 1 and 2). It should be noted that the effect of the F4.3S product in reducing stress phytotoxicity was more significant under drought stress, which in this experiment was characterized as being the most harmful to zucchini plants. Therefore, in moderate stresses (in our case cold stress) and severe stresses (in our case drought stress), the application of F4.3S can be defined as a biostimulant product with a protective and detoxifying effect that allows the improvement of the recovery of plants once the adverse conditions for growth are over. This protective effect of F4.3S could be due to the action of various compounds added to the biostimulant. Thus, it was observed that plants subjected to stress and treated with compounds such as allantoin, salicylic acid, certain amino acids, and elements such as Se and Mo showed increased stress tolerance and consequently greater biomass (Campobenedetto et al., 2020; Carillo et al., 2019; Deng et al., 2020; Kaur et al., 2021; Medrano-Macías and Narvaéz-Ortiz, 2022).

In addition to the study of biomass, the analysis of leaf RWC is considered a reliable indicator that reflects the plant's ability to return to a favorable water state after a water deficit and its tolerance capacity against this type of stress. It was proved that cold stress and water deficit cause a decrease in the RWC of leaves in most sensitive plants, which has defined this indicator as a factor for identifying tolerant and sensitive genotypes (Ilyas et al., 2021; Novák et al., 2021; Pour-Aboughadareh et al., 2019). In the present study, only plants subjected to water stress significantly reduced leaf RWC, with no changes appearing between the values of RWC in the case of cold stress (Table 2). The application of the F4.3S product led to a restoration of leaf RWC with values similar to those of non-stressed control plants (Table 2), which clearly indicates the beneficial effect of applying this product in conditions of water deficit.

Furthermore, under adverse conditions, such as cold and drought stress, maintaining the integrity of cell membranes is crucial for plant survival. The efflux or leakage of electrolytes from the cell has been used as another indicator of damage to cell membranes (He et al., 2018; Ilyas et al., 2021; Navarro-León et al., 2020; Novák et al., 2021). As occurred with leaf RWC, changes were only observed in the case of drought stress, with no differences appearing between treatments for cold stress (Table 2). In drought stress, the EL through the cell membranes occurred with high values in the plants subjected to this type of stress, although it should be noted that the application of the product F4.3S decreased EL

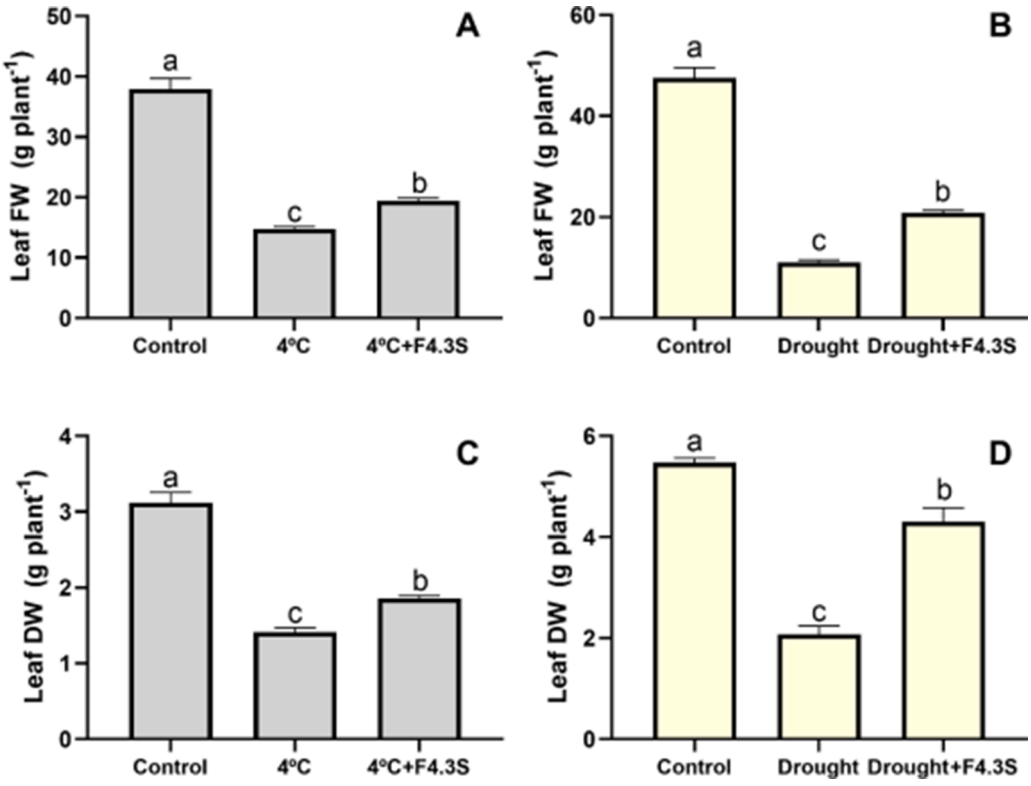

Fig. 2. Biomass production of fresh and dry shoot part in the drought stress test at the time of plant sampling.

Table 2
Relative leaf water content (RWC) and electrolyte leakage (EL) in cold and drought stress tests at the time of plant sampling.

Treatments	RWC (%)	EL (%)
Control	80.22 ± 3.64[a]	19.45 ± 1.66[a]
4 °C	83.64 ± 1.48[a]	19.06 ± 2.60[a]
4 °C + F4.3S	80.20 ± 1.41[a]	19.79 ± 0.49[a]
p-value	N.S.	N.S.
Control	79.53 ± 1.72[a]	15.00 ± 2.96[b]
Drought	72.88 ± 0.61[b]	30.75 ± 1.32[a]
Drought + F4.3S	78.30 ± 3.01[a]	20.46 ± 1.03[b]
p-value	*	**

Values are means ± standard error. Significance levels are represented by N.S. (not significant) $P > 0.05$; * $P < 0.05$ and ** $P < 0.01$.

with values lower than those obtained in stressed plants without application of the product (Table 2). These results show the detoxifying and protective effect of the F4.3S product, partly restoring the integrity of cell membranes after drought stress has passed. In other experiments, the application of biostimulants containing compounds such as ascorbate, salicylic acid, proline, and Se was also effective in increasing RWC and reducing EL in drought-stressed plants (Abdali et al., 2023; Rady et al., 2020; Semida et al., 2020).

3.2. Stress indicators

The increase in MDA concentration in plants is also related to membrane destabilization. Thus, MDA is the indicator parameter of membrane lipid peroxidation, and an increase in its values suggests an excessive presence of ROS. The reduction of ROS accumulation is crucial for the survival of plants under cold and water-limiting conditions, which is why the study of oxidative metabolism has been used as an indicator of the damage caused by these types of stress (Nxele et al., 2017; He et al., 2018; Hasanuzzaman et al., 2021). One of the possible protective effects of biostimulants against abiotic stresses is mainly because these products reduce cellular oxidative damage, and thereby the peroxidation of membrane lipids, by regulating antioxidant defense and decreasing ROS levels (Van Oosten et al., 2017; Bulgari et al., 2019; Rajabi Hamedani et al., 2020). In our experiment, plants that presented the highest biomass production showed the lowest MDA levels, that is, non-stressed control plants and plants with cold stress and water stress together with the application of the product F4.3S (Table 3). Similar to MDA, the maximum foliar concentrations of H_2O_2 and O_2^- were observed in plants subjected to cold stress and drought stress, whereas the application of the F4.3S product reduced both types of stress, except for H_2O_2 in plants subjected to cold stress. The most significant reduction was observed in the case of drought stress (Table 3). Thus, the oxidative stress data support the positive, protective, and detoxifying effects of the F4.3S product under cold stress conditions and fundamentally under drought conditions. Several of the F4.3S components

Table 3
Indicators of oxidative stress and proline concentration in the cold and drought stress tests at the time of plant sampling.

Treatments	**MDA** (μM g^{-1} FW)	**O_2^-** (μg g^{-1} FW)	**H_2O_2** (μg g^{-1} FW)	**Proline** (μg g^{-1} FW)
Control	2.75±0.04[c]	3.63 ± 0.19[c]	67.90 ± 13.94[b]	46.72 ± 1.85[c]
4 °C	8.36±0.14[a]	6.58±0.45[a]	163.90±10.97[a]	56.67 ± 1.98[b]
4 °C + F4.3S	5.32 ± 0.20[b]	5.05 ± 0.33[b]	146.07 ± 19.33[a]	69.77± 4.21[a]
p-value	***	**	*	*
Control	2.84 ± 0.06[b]	2.38 ± 0.11[c]	70.06±8.44[c]	45.90 ± 3.72[c]
Drought	5.97±0.29[a]	7.43±0.64[a]	259.98±10.90[a]	59.14 ± 2.34[b]
Drought + F4.3S	3.63 ± 0.60[b]	3.45 ± 0.21[b]	87.74 ± 10.52[b]	84.24±1.92[a]
p-value	***	***	***	***

Values are means ± standard error. Significance levels are represented * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$.

have antioxidant-defense-promoting effects, such as allantoin, certain amino acids, ascorbate, and Se, which have been shown in other studies to reduce the accumulation of ROS in plants subjected to various abiotic stresses (Rady et al., 2020; Kaur et al., 2021; Medrano-Macías and Narvaéz-Ortiz, 2022; Navarro-León et al., 2022; Abdali et al., 2023).

Proline plays an osmoprotective, osmoregulatory, and antioxidant role against ROS accumulation (Kaur and Asthir, 2015). In the present study, proline concentration increased compared with that obtained in control plants in all stress treatments, suggesting a possible tolerance response to adverse growth conditions. It should be noted that the proline concentration presented a very significant increase in the most tolerant plants, that is, in those to which the F4.3S product was applied, and especially in the case of drought stress (Table 3). This increase may be due either to proline endogenously synthesized by the plant or incorporated from the F4.3S product because it is one of its components. Furthermore, the accumulation of proline under cold and drought stress conditions due to the application of the F4.3S product could be responsible in these plants for both the improvement of membrane integrity and the increase in leaf RWC (Table 2). Other studies in plants subjected to water and cold stress also proved the beneficial effects of the application of external proline because of their osmoprotective and antioxidant properties (Semida et al., 2020; Uzal, 2022).

3.3. Photosynthetic parameters

In general, significant inhibition of photosynthesis occurs in plants under environmental stress (Jordan-Meille et al., 2018; Navarro-León et al., 2020; Ilyas et al., 2021). It was shown in some plant species that the application of biostimulants reverses this inhibition and therefore restores normal plant growth (Van Oosten et al., 2017; Bulgari et al., 2019; Rajabi Hamedani et al., 2020). To verify the possible positive effect of recovery and/or detoxification of the F4.3S product once the cold and drought stress has ended, we analyzed the response of the photosynthetic process in plants by studying different parameters that directly define the photosynthetic activity, such as photochemical activity through Chl *a* fluorescence, the concentration of photosynthetic pigments, and the photosynthetic gas exchange parameters.

It was proven that Chl *a* fluorescence reflects the photosynthetic state of the plant and the photosynthetic changes produced under the effects of stress (Strasser et al., 2000). One of the parameters derived from the analysis of Chl *a* fluorescence is the quantum yield of primary photosynthesis (Fv/Fm), which is a good indicator of the photosynthetic performance of plants. In healthy plants not subjected to stress, the Fv/Fm value is usually around 0.85 (Abdeshahian et al., 2010). Table 4 shows that control zucchini plants presented Fv/Fm values similar to 0.85, whereas the plants subjected to the two applied stresses showed significantly lower values, regardless of the application or not of the F4.3S product, indicating greater Chl *a* fluorescence and therefore a greater degree of stress than control plants. Regarding the remaining parameters, a reduction in the values of the photosynthetic performance index (PIabs) and the output of electrons mainly from photosystem II (ψ_{Eo}) in plants subjected to both stresses was observed, whereas the proportion of active reaction centers (RC/ABS) was reduced only in plants subjected to cold stress. The application of the F4.3S product did not increase the values of these indices compared with those of the stressed plants, although in the case of the plants stressed by cold, the RC/ABS and PIabs values did not show significant differences compared with the control plants (Table 4). Hence, the photochemical activity and vitality of the plants through the functioning of the photochemical phase of photosynthesis, suggest that under cold and drought conditions the application of the F4.3S product does not imply a significant improvement from a photochemical point of view of the different components of the photochemical stage, since the values of these indices were similar among the stressed plants regardless of the application of the F4.3S product (Table 4).

In addition to the parameters of Chl *a* fluorescence, the concentrations of Chl *a, b*, and carotenoids are indicative of photosynthetic activity (Li et al., 2010). As observed in Table 5, the values of photosynthetic pigments increased under conditions of cold and drought stress with the application of the F4.3S product. Thus, these plants presented higher values than plants subjected to both stresses and without the application of the product. Regarding the maximum values of these pigments, as expected, they occurred in control plants not subjected to any type of stress (Table 5). These results suggest that under cold and drought stress conditions, the foliar application of F4.3S could improve the light energy capture capacity. Concerning carotenoids, these pigments are integral components of the thylakoid membranes within chloroplasts and function both as accessory light-harvesting pigments and as antioxidants, scavenging reactive oxygen species (ROS) during plant stress (Havaux, 1998). This dual role could account for the protective effect on the photosynthetic apparatus observed following the application of the F4.3S product to zucchini plants exposed to cold and drought stress. Notably, the treated plants exhibited significantly higher carotenoid values than those subjected solely to stress (Table 5). Other studies observed that the application of certain bioactive compounds present in F4.3S such as free AAs and Se enhanced carotenoid accumulation in cabbage and tomato plants (Garza-García et al., 2023; Haghighi et al., 2022).

Gas exchange parameters are indicative of photosynthetic efficiency and are determining factors in the adaptation of plants to any type of stress (Zhao et al., 2015; Maghsoudi et al., 2016). When plants were exposed to cold and drought stress, leaf water loss decreases through a significant reduction in *E* due to higher r. Thus, stomatal closure is considered a rapid adaptation mechanism to these types of abiotic stresses and is essential for reducing plant water loss. However,

Table 4
CHL *a* fluorescence parameters in the cold and drought stress tests at the time of plant sampling.

Treatment	Fv/Fm	RC/ABS	PI (Abs)	ψEo
Control	0.843 ± 0.002^{a}	0.48 ± 0.01^{a}	3.03 ± 0.06^{a}	0.57 ± 0.01^{a}
4 °C	0.831 ± 0.002^{b}	0.43 ± 0.01^{b}	2.43 ± 0.12^{b}	0.54 ± 0.01^{b}
4 °C + F4.3S	0.828 ± 0.001^{b}	0.46 ± 0.02^{ab}	2.69 ± 0.16^{ab}	0.53 ± 0.01^{b}
p-value	*	*	*	*
Control	0.853 ± 0.001^{a}	0.51 ± 0.01^{a}	3.46 ± 0.09^{a}	0.59 ± 0.004^{a}
Drought	0.824 ± 0.003^{b}	0.47 ± 0.02^{a}	2.52 ± 0.23^{b}	0.53 ± 0.01^{b}
Drought + F4.3S	0.820 ± 0.002^{b}	0.47 ± 0.01^{a}	2.47 ± 0.16^{b}	0.54 ± 0.01^{b}
p-value	*	N.S.	**	***

Values are means ± standard error. Significance levels are represented by N.S. (not significant) $P > 0.05$; * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$.

Table 5
Photosynthetic pigments in cold and drought stress tests at the time of plant sampling.

Treatment	Chl *a* (mg g^{-1} FW)	Chl *b* (mg g^{-1} FW)	Carotenoids (μg g^{-1} FW)
Control	0.77 ± 0.02^{a}	0.38 ± 0.01^{a}	96.58 ± 2.90^{a}
4 °C	0.61 ± 0.01^{c}	0.34 ± 0.01^{b}	68.93 ± 4.00^{c}
4 °C + F4.3S	0.68 ± 0.01^{b}	0.366 ± 0.004^{a}	80.40 ± 2.38^{b}
p-value	***	***	***
Control	0.91 ± 0.04^{a}	0.43 ± 0.01^{a}	130.71 ± 8.95^{a}
Drought	0.55 ± 0.02^{c}	0.32 ± 0.01^{c}	64.82 ± 4.30^{c}
Drought + F4.3S	0.71 ± 0.02^{b}	0.38 ± 0.01^{b}	91.95 ± 2.14^{b}
p-value	***	***	***

Values are means ± standard error. Significance levels are represented by *** $P < 0.001$.

long-term maintenance of this strategy is generally counterproductive because stomatal closure reduces CO_2 entry, leading to a reduction in photosynthesis and therefore to the lack of the endogenous electron acceptor NADP, which ultimately results in the formation of ROS (Nxele et al., 2017). In our study, both stress conditions reduced the *A* parameter, although the application of the F4.3S product reversed this effect. Notably, in drought-stressed plants treated with F4.3S, higher levels of *A* were observed compared with the control plants. In terms of *E*, the application of the F4.3S product increased its value while reducing r, whereas the other treatments did not show significant differences. Lastly, regarding WUE, both stress conditions decreased its value, although, in the case of water stress, the application of F4.3S was beneficial, increasing the value of this parameter compared with the stressed plants without the product (Table 6). Different studies have indicated that the application of certain biostimulants under stress conditions could prevent a total closure of the stomata under stress conditions, which would favor the maintenance of photosynthetic activity in plants, thus reducing the generation of massive ROS under these conditions (Van Oosten et al., 2017; Semida et al., 2020). The data obtained in the present study confirm that the application of the F4.3S product would enhance photosynthetic performance and, in the case of drought stress, increase WUE once this type of stress is over (Table 6). Specifically, other studies observed that certain compounds applied to plants, such as proline, ascorbate, salicylic acid, Co, and Se, contribute to the maintenance of photosynthesis components and increase WUE under cold and water stress conditions (Semida et al., 2020; Chongping et al., 2022; Abdali et al., 2023; Elshamly, 2023). Specifically, the increased proline content in zucchini plants supplied with F4.3S could enhance osmoprotection, maintaining the RWC, and thus allowing greater stomatal opening for CO_2 assimilation, which increases plant growth (Kimura et al., 2020).

3.4. Ethylene response

One of the molecules that determines the appearance of any type of abiotic stress is ethylene (Fatma et al., 2022). Examples of this phytotoxic aspect of ethylene under abiotic stress conditions are abundant in the literature, and stress-tolerant plants typically exhibit lower levels of this hormone (Zapata et al., 2007; Siddikee et al., 2012). In the present experiment, the effectiveness of the product F4.3S in reducing ethylene synthesis was tested through the analysis of ACC concentration and the activity of ACC oxidase, one of the key enzymes in the synthesis of this hormone. Thus, plants stressed by cold and drought showed higher values of these parameters without observing statistically significant differences between plants treated with F4.3S and those not treated, although it should be noted that the application of F4.3S did reduce both ACC concentration and ACC oxidase activity (Table 7). The application of the F4.3S product reduced ethylene synthesis, a mechanism that appears to be more effective throughout the stress period. This could account for the enhanced recovery of the plants post-stress, as observed in this study. It is conceivable that the protective effect of the F4.3S product dissipates once stress conditions cease and better growth conditions are restored, thereby explaining the absence of significant differences in the ACC and ACC oxidase parameters between the stressed plants.

Table 6
Gas exchange parameters in cold and drought stress tests at the time of plant sampling.

Treatment	*A* (μmol m^{-2} s^{-1})	*E* (mmol m^{-2} s^{-1})	r (s cm^{-1})	WUE (μmol $mmol^{-1}$)
Control	5.37 ± 0.82^{a}	1.22 ± 0.13^{b}	22.27 ± 2.40^{a}	4.39 ± 0.09^{a}
4 °C	3.28 ± 0.35^{b}	1.12 ± 0.13^{b}	25.25 ± 2.70^{a}	3.05 ± 0.31^{b}
4 °C + F4.3S	5.38 ± 0.99^{a}	1.76 ± 0.24^{a}	16.19 ± 2.20^{b}	2.96 ± 0.16^{b}
p-value	*	*	*	***
Control	3.39 ± 0.23^{b}	0.95 ± 0.10^{b}	29.66 ± 3.13^{a}	3.66 ± 0.45^{a}
Drought	1.90 ± 0.22^{c}	1.10 ± 0.11^{b}	27.44 ± 3.32^{a}	1.73 ± 0.09^{c}
Drought + F4.3S	4.38 ± 0.19^{a}	1.68 ± 0.10^{a}	16.61 ± 1.27^{b}	2.63 ± 0.06^{b}
p-value	***	***	**	***

Values are means ± standard error. Significance levels are represented by * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$.

4. Conclusions

Under cold and drought stress conditions, the application of the F4.3S product shows a detox or recovery effect in zucchini plants once these stresses are over because it gives rise to a significant increase in plant growth. The combination of bioactive compounds in the biostimulant improved the physiological condition of zucchini plants and increased the proline and carotenoid contents, which could limit ROS accumulation and provide osmoprotection. Consequently, this beneficial effect facilitated a greater opening of stomata, thereby allowing enhanced CO_2 assimilation, which played a significant role in promoting growth. Under conditions of water stress, these positive effects of F4.3S led to a higher WUE, which was crucial in the tolerance to this type of stress. The implications of this study underscore the potential of F4.3S as a means to bolster plant resilience against abiotic stresses, which may be particularly pertinent in the face of escalating climatic challenges.

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CRediT authorship contribution statement

Iván Navarro-Morillo: Methodology, Formal analysis. **Eloy Navarro-León:** Writing – original draft, Formal analysis. **Santiago Atero-Calvo:** Methodology. **Juan José Rios:** Methodology, Formal analysis, Data curation. **Juan Manuel Ruiz:** Writing – review & editing, Conceptualization. **Begoña Blasco:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Iván Navarro-Morillo certifies that he is employed by Atlántica Agrícola S.L. and hereby declares that he provided the biostimulant used in this experiment, as well as participated in writing and editing this manuscript. The remaining authors declare no conflict of interest. The

Table 7
Ethylene synthesis parameters in cold and drought stress tests at the time of plant sampling.

Treatment	ACC (ng g^{-1} DW)	ACC oxidase (nmol mg $protein^{-1}$ min^{-1})
Control	14.46 ± 2.23^{b}	0.27 ± 0.03^{b}
4 °C	22.05 ± 3.35^{a}	0.41 ± 0.06^{a}
4 °C + F4.3S	19.38 ± 2.99^{a}	0.35 ± 0.09^{a}
p-value	*	*
Control	13.69 ± 1.29^{b}	0.35 ± 0.09^{b}
Drought	35.90 ± 4.02^{a}	0.90 ± 0.21^{a}
Drought + F4.3S	31.24 ± 4.19^{a}	0.78 ± 0.20^{a}
p-value	*	*

Values are means ± standard error. Significance levels are represented by * $P < 0.05$.

biostimulant is not a commercial product at the time of publication of this article.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2024.113114.

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