



Research article

Fermentation-derived biostimulant BiH2-B enhances abiotic stress tolerance in horticultural crops

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Abstract: Abiotic stresses such as transplant shock and low-temperature events represent major constraints to early crop establishment and productivity in horticultural systems. This study evaluated the effect of the fermentation-derived biostimulant BiH2-B obtained from the exhausted fermentation broth of dark biohydrogen production (BiH2 technology, Biorenova S.p.A.) in mitigating transplant and chilling related stress in tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annuum* L.) grown in climatic chambers conditions. In tomatoes, five BiH2-B doses (10–25 L ha⁻¹) were applied via fertigation at and after transplanting; growth and physiological parameters were monitored during early vegetative development. BiH2-B significantly increased leaf chlorophyll (SPAD) and leaf area up to 15 L ha⁻¹, with a maximum leaf area increase of 7.5% and higher aerial fresh biomass at intermediate doses, without affecting the root biomass or inducing phytotoxicity. In peppers, the same application rates (10–25 L ha⁻¹) were foliar-applied as foliar sprays before and after an imposed chilling event (6 °C for 48 h). Intermediate rates (12–15 L ha⁻¹) enhanced SPAD (+10.3%) and plant height (7.4%) and markedly reduced chilling injury compared with the untreated control, while higher rates did not provide additional benefits. Overall, these results identify an optimal application range for BiH2-B between 12 and 15 L ha⁻¹ and support the use of fermentation-derived biostimulants as sustainable tools to enhance physiological resilience to abiotic stress in horticultural crops, without exerting a fertilizing effect and in line with the European Union (EU) definition of plant biostimulants.

Keywords: fermentation-derived biostimulant; abiotic stress tolerance; BiH2-B; horticultural crops; tomato; pepper; circular economy; cold stress; post-transplant stress

1. Introduction

Climate change is increasingly intensifying the frequency and severity of abiotic stresses such as drought, salinity and extreme temperatures, which collectively represent one of the main causes of yield instability in horticultural production systems worldwide [1,2]. In addition to the direct effects of these stressors on plant physiology, climate change is expected to exacerbate the prevalence, severity, and frequency of pest infestations and disease outbreaks [3,4]. According to recent estimates, abiotic stress contributes to 50% of global agricultural productivity losses [5]. These losses, combined with the ongoing increase in human population, emphasize the need to improve agricultural productivity and resilience [6]. In this context, plant biostimulants have emerged as valuable tools to enhance crop resilience to abiotic stresses while reducing reliance on conventional agrochemical inputs [7]. According to European Regulation (EU) 2019/1009, “plant biostimulants are products that stimulate plant nutrition processes independently of their nutrient content, with the aim of improving nutrient use efficiency, tolerance to abiotic stress, quality traits or nutrient availability in the rhizosphere.” [8]. A wide range of biostimulant categories have been identified, including humic substances, protein hydrolysates, amino acids, seaweed extracts, microbial inoculants, and fermentation-derived products [9,10]. Numerous studies have demonstrated that these products can enhance the chlorophyll content, photosynthetic efficiency, biomass accumulation, and stress tolerance under suboptimal growing conditions in horticultural crops [11,12]. Among these, microbial and fermentation-based biostimulants are gaining increasing attention due to their complex composition of low-molecular-weight bioactive compounds, such as amino acid, oligopeptides, and organic acids, which can modulate plant physiological and metabolic responses [13,14]. Several authors have reported that such products can stimulate the primary metabolism, enhance nitrogen uptake and assimilation, and activate stress-responsive pathways, thereby improving plant performance under abiotic stress condition [15,16]. In particular, fermentation-derived products have been shown to promote faster recovery of photosynthetic activity after stress events, improve canopy development, and strengthen plant defense mechanisms against temperature, salinity and drought stress [17,18]. The increasing interest in fermentation-derived biostimulants is also aligned with circular bioeconomy strategies, in which biorefinery processes can generate bioactive co-products suitable for agricultural valorization [19]. This approach is particularly relevant for dark fermentation technologies, where biohydrogen production is accompanied by the formation of exhausted fermentation broths containing low-molecular-weight compounds with potential plant biostimulant activity [20,21]. The BiH2 technology (Biorenova S.p.A.), patented in Italy (Grant Number IT102020000013006), Saudi Arabia (Grant Number 14842) and subject of the international PCT application PCT/EP2021/063588, represents an innovative biorefinery platform which integrates biohydrogen production, CO₂ absorption, and the generation of high value co-products. Phase 1 of the BiH2 process (P1) is based on a dark fermentation step using *Clostridium beijerinckii*, which yields an exhausted fermentation broth rich in low-molecular-weight compounds with potential biostimulant activity. Recent research demonstrated that an exhausted fermentation broth obtained from the BiH2 P1 process, previously referred to as BEFB, exhibited significant biostimulant effects under field conditions [22]. In apple orchards, BEFB application improved the fruit quality parameters, thus supporting the agronomic potential of fermentation-derived-co-products from biohydrogen production chains [22]. Building on these findings, the formulation referred to as

BiH2-B was developed through an improved filtration and stabilization process, yielding a cell-free microbial-biostimulant with a chemical profile comparable to BEFB and suitable for controlled agricultural application. Despite the growing interest in fermentation-derived biostimulants, limited information is available on their efficacy under controlled environmental conditions and their specific role in mitigating abiotic stress factors at early developmental stages. Despite the growing number of studies on plant biostimulants, physiological evaluations under controlled stress conditions remain essential to isolate stress-specific responses prior to mechanistic or field-scale investigations.

Tomatoes (*Solanum lycopersicum* L.) and peppers (*Capsicum annuum* L.) are highly responsive horticultural crops for evaluating biostimulant efficacy under abiotic stress. In tomato, fermentation-derived or hydrolysate-based biostimulants have been shown to improve morpho-physiological traits, photosynthetic activity, and post-stress recovery, thus supporting their use under suboptimal conditions such as drought or transplant-related stress [23–25]. Similarly, peppers, as a thermophilic species highly sensitive to salinity, drought, and low-temperature events, has shown positive responses to microbial and protein-hydrolysate biostimulants, including improved growth, chlorophyll accumulation, and stress recovery [26–28]. These findings indicate that both tomatoes and peppers can benefit from biostimulants rich in microbial or fermentation-derived bioactive compounds and provide a strong rationale for testing BiH2-B, a cell-free fermentation-derived biostimulant, under controlled post-transplant and cold stress conditions. Therefore, the present study aimed to evaluate the efficacy of the fermentation-derived-biostimulant BiH2-B in mitigating abiotic stress in horticultural crops under climatic chamber conditions. This study focuses on physiological outcomes under controlled conditions as a necessary step prior to large-scale field validation. Specifically, the objectives were as follows: (i) Assess the ability of BiH2-B to alleviate post-transplant stress in tomatoes (*Solanum lycopersicum* L.); and (ii) evaluate its effectiveness in reducing chilling injury in peppers (*Capsicum annuum* L.). The working hypothesis was that BiH2-B would enhance chlorophyll-related parameters, canopy development, and stress recovery in a dose-dependent manner.

2. Materials and methods

2.1. BiH2-B production process

The microbial biostimulant BiH2-B, referred to as BEFB in previous research [22], was produced according to P1 of the BiH2 technology as described by Galiano et al. [22]. P1 consists of a fed-batch fermentation process corresponding to the biohydrogen production stage of the BiH2 technology (Biorenova S.p.A.) carried out using *Clostridium beijerinckii* DSM 34075. At the end of the fermentation process, the exhausted broth was harvested and filtered through a 0.22 µm pore size Millipore filter (Sigma-Aldrich, St. Louis–MO, USA) to obtain a cell-free product, which was subsequently stored at 4 °C until its application. As reported in a previous study [22], BiH2-B is characterized by a high content of bioactive low-molecular-weight compounds, particularly oligopeptides and organic acids, which are associated with its biostimulant activity. Chemical analyses confirmed that the BiH2-B product exhibited a composition comparable, in terms of concentration and profile of bioactive compounds, to the BEFB previously tested [22]. Moreover, the adoption of an improved cellular filtration process during BiH2-B production enhanced the stability of the resulting biostimulant formulation.

2.2. Growth chamber experiment

The biostimulant activity of BiH2-B on vegetable crops was evaluated through two independent experiments specifically designed to assess its effect on abiotic stress conditions under controlled growth chamber environments. Both trials were conducted in a climatic chamber by a certified research organization (Res Agraria S.r.l., Tortoreto Lido, Teramo, Italy), under Mediterranean environmental conditions (EPPO code EPOMED), allowing precise control of the temperature, light and humidity in order to isolate plant responses to abiotic stress. The use of controlled environmental conditions allowed for the isolation of plant physiological responses to specific abiotic stress factors, thus minimizing confounding environmental variability.

The experiment focused on two major abiotic stress scenarios relevant to horticultural production: post-transplant stress (*Solanum lycopersicum* L. TR-1) and cold stress tolerance in peppers (*Capsicum annuum* L. TR-2). In both trials, five application volumes of BiH2-B (10 L ha^{-1} , 12 L ha^{-1} , 15 L ha^{-1} , 20 L ha^{-1} , 25 L ha^{-1}) labeled as BiH2-B1, BiH2-B2, BiH2-B3, BiH2-B4, and BiH2-B5, respectively, were tested alongside an unfertilized and untreated controls (CTR). To ensure that observed plant responses were exclusively attributable to the biostimulant treatment, no basal fertilizer or additional nutrients inputs were applied during the experimental period. Moreover, all treatments, including the control, received identical water volumes, thus excluding any confounding effects related to differential irrigation or spray volume. In both trials, the experimental unit was the plot, and all statistical analyses were conducted using plot means derived from individual plant measurements.

2.2.1. Post-transplant stress and roots expansions on tomato trial (TR-1) experimental design

The tomato trial was conducted from June 1st to June 27th, 2022, at Res Agraria S.r.l., (Tortoreto Lido-TE, Italy). The growth chamber was maintained at a constant light intensity of 2000 lux. The temperature and relative humidity were recorded daily throughout the experiment (Figure 1).

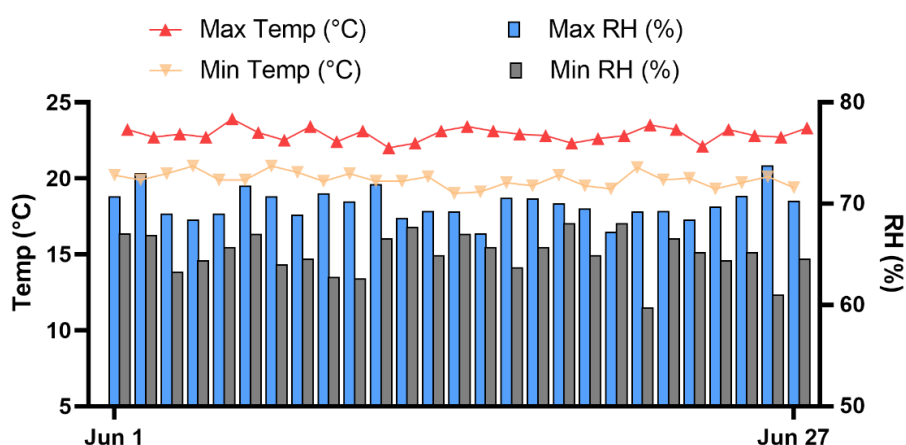


Figure 1. Temperature (Min and Max) and relative humidity (Min and Max) recorded in climatic chamber during post-transplant stress trial on *Solanum lycopersicum* L. (TR-1).

The tomato plants ('ROMANELLO' F1 hybrids) were transplanted and grew in a peat substrate (40% organic matter, pH 6.0). No basal fertilization was applied before or during the experiment. The experimental unit consisted of a plot of five plants, the trial included four replicated plots per treatment,

each plot consisting of five plants ($n = 4$ plots per treatment). BiH2-B treatments were applied by drip irrigation at three time points: (A) At transplanting (June 1st, BBCH 12-13), (B) 7 days after transplant (June 8th, BBCH 22-23), and (C) at BBCH 51-52 (June 15th). Applications were performed using a water volume of 300 mL per plant, corresponding to 20000 L ha⁻¹. The control treatment (CTR) received the same irrigation schedule and the same volume of water per plant, without the addition of biostimulants, thus ensuring that any observed differences among treatments were exclusively attributable to the BiH2-B application. Treatments were assigned according to randomized complete block design with four replicates. Each plot consisted of five plants spaced 0.15 m x 0.3 m for a total of 120 plants. The following parameters were evaluated: leaf chlorophyll, plant stress symptoms, plant height, leaf area, fresh and dry biomass, tissue damage (%), and phytotoxicity. Leaf chlorophyll was measured using a SPAD-502 Meter (Minolta, Japan). Plant vigor was assessed using a GreenSeeker Handheld Crop Sensor (Trimble, USA), which estimates crop vigor and biomass through measurement of the Normalized Difference Vegetation Index (NDVI), calculated using reflectance values in the red (600 nm) and near-infrared (NIR, 800 nm) spectral regions. Plant height and leaf area measured with Petiole Pro. Measurements were performed on all 120 plants at three times: 6th June (BBCH 14), 17th June (BBCH 52), and 27th June (BBCH 71).

2.2.2. Cold stress on pepper (TR-2) experimental design

The cold stress trial was carried out in a growth chamber under controlled environmental conditions to evaluate the effects of BiH2-B on pepper plants subjected to low-temperature stress. The experiment lasted from June 28th to July 16th 2022. The growth chamber light intensity was maintained at 2000 lux, temperature ranged between 19–23 °C under normal conditions, and relative humidity varied from 61% to 74% (Figure 2). Pepper plants ('TRIVIO' F1 hybrid) were transplanted at the 5–7 true leaf stage into a peat-based substrate (90% organic matter, pH 6.0). No basal fertilization was applied during the experiment. The experimental design was a randomized complete block with four replicates, where each treatment plot consisted of three plants ($n = 4$ plots per treatment), spaced 0.15 m x 0.30 m, for a total 72 plants. Five BiH2-B application volumes were tested alongside an untreated control (CTR). Treatments were applied as foliar sprays using a volume of 1.3 mL per plant, corresponding to a spray volume of 1000 L ha⁻¹. Both the BiH2-B treatments and the control received the same spray volume, with control plants treated only with water. Applications were performed at two time points: (A) 24 h before and (B) 24 h after an imposed cold stress event (29th June, BBCH 15-16), during which the growth chamber temperature was reduced to 6 °C for 48 h, though all the other parameters of the climatic chamber remained unchanged.

Leaf chlorophyll, plant vigor, plant height, and tissue damage were assessed on all plants. Leaf chlorophyll was measured using a SPAD-502 meter (Minolta, Japan). Plant vigor was evaluated with a GreenSeeker Handled Crop Sensor (Trimble, USA) by measuring the NDVI, calculated from red (600 nm) and near-infrared (800nm) reflectance, while plant height was determined using Petiole Pro. Measurements were performed on all plants five times: 28th June (BBCH 15), 1st July (BBCH 16), 3rd July (BBCH 17), 6th July (BBCH 18), and 16th July (BBCH 44).

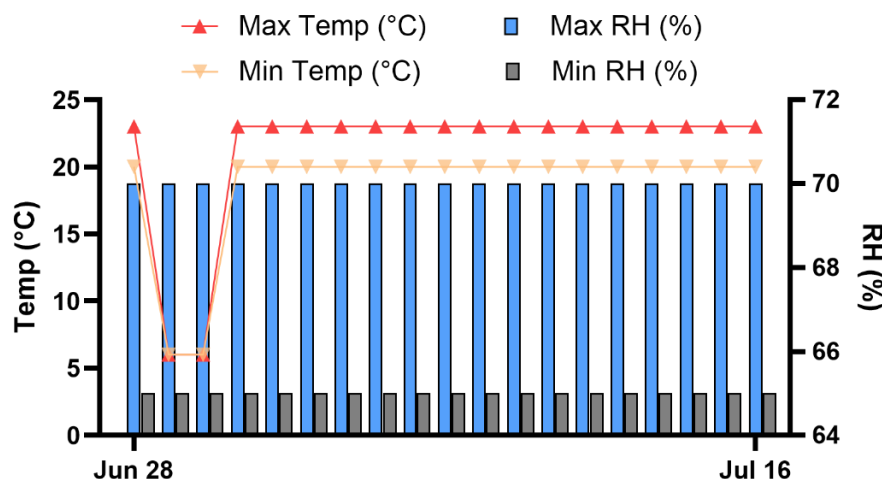


Figure 2. Temperature (Min and Max) and relative humidity (Min and Max) recorded in climatic chamber during cold stress trial on *Capsicum annuum* L. (TR-2).

2.2.3. Statistical analysis

Plant growth and physiological parameters were analyzed using one-way analysis of variance (ANOVA). When the treatment effects were significant ($p < 0.05$), means were separated using Student–Newman–Keuls (SNK) post-hoc test, which is appropriate for dose–response experiments involving multiple treatment levels and has been widely used in biostimulant research. Statistical analyses were performed using the ARM software (GDM Solutions, Inc., Brookings, SD, USA). Results are reported as means $\pm$ standard deviation (SD).

3. Results

3.1. Tomato trial

To evaluate the effect of the BiH2-B on the tomato plants physiology, parameters such as SPAD, NDVI, plant height, and leaf area were measured through two intermediate assessments (Tables 1 and 2); furthermore, at final assessment, quantitative parameters were also evaluated such as fresh and dry aerial biomass, and fresh and dry roots biomass (Tables 3 and 4). Results in Tables 1–4 show how BiH2-B applied at different rates can significantly ($p < 0.05$) improve the physiological performance and growth of tomato plants under transplant-related stress. The SPAD value and leaf area were linearly improved by increasing rates of BiH2-B up to 15 L ha⁻¹.

With BiH2-B3 application, the leaf area showed an increase of 7.5% with respect to the untreated CTR; similar responses were recorded also with the SPAD measurement. Instead, the aerial fresh biomass was significantly higher than the untreated control with 12 L ha⁻¹ of BiH2 (B2). Higher rates (20–25 L ha⁻¹) did not outperform the 15 L ha⁻¹; they gave slightly diminished responses regarding some parameters. Therefore, under the tests conditions, the medium rate of BiH2-B appeared the best dosage to improve the vegetative growth and photosynthetic activities of the tomato plants. These results indicate that BiH2-B can significantly reduce transplant stress in tomatoes in its first stage of growth. However, no phytotoxic symptoms were observed across any treatment, even at high rates. The fermented broth showed an excellent solubility and was easily applied via irrigation.

Table 1. Parameters measured at first assessment (6 June, BBCH 14) in post-transplant stress on *Solanum lycopersicum* L. (TR-1).

Treatment	SPAD value (SD)	NDVI (SD)	Plant Height cm (SD)	Leaf Area cm ² (SD)
CTR	11.05 (± 2.01)	0.63 (± 0.01)	17.75 (± 0.83)	67.58 (± 1.05) d
BiH2-B1	11.55 (± 0.39)	0.63 (± 0.06)	18.58 (± 0.96)	74.08 (± 1.38) bc
BiH2-B2	11.80 (± 0.64)	0.63 (± 0.02)	18.00 (± 1.21)	75.78 (± 2.25) b
BiH2-B3	12.53 (± 0.48)	0.62 (± 0.02)	18.50 (± 2.25)	84.28 (± 2.53) a
BiH2-B4	11.83 (± 1.22)	0.64 (± 0.03)	18.00 (± 2.25)	71.90 (± 1.20) c
BiH2-B5	11.35 (± 0.72)	0.62 (± 0.04)	18.55 (± 0.86)	73.33 (± 1.54) bc
Significance	ns	ns	ns	*

*Note: SPAD value = Relative chlorophyll content measured using SPAD-502 Meter (Minolta, Japan); NDVI = Normalized Difference Vegetation Index; SD = standard deviation; CTR = control; BiH2-B1 = (10 L ha⁻¹); BiH2-B2 (12 L ha⁻¹); BiH2-B3 (15 L ha⁻¹); BiH2-B4 (20 L ha⁻¹); BiH2-B5 (25 L ha⁻¹); ns = no significant differences among treatments (one-way ANOVA); * = significant differences among treatments at the $p < 0.05$ level (one-way ANOVA); different lowercase letters indicate significant differences among treatments at the $p < 0.05$ level (Student-Newman-Keuls post-hoc test).

Table 2. Parameters measured at second assessment (17 June, BBCH 52) in post-transplant stress on *Solanum lycopersicum* L. (TR-1).

Treatment	SPAD value (SD)	NDVI (SD)	Plant Height cm (SD)	Leaf Area cm ² (SD)
CTR	36.38 (± 0.59)	0.78 (± 0.01) b	25.28 (± 0.77)	123.79 (± 0.40) d
BiH2-B1	37.34 (± 4.64)	0.78 (± 0.01) b	25.44 (± 1.58)	126.29 (± 2.83) bc
BiH2-B2	37.10 (± 0.82)	0.78 (± 0.01) b	25.08 (± 1.21)	127.36 (± 0.32) b
BiH2-B3	39.16 (± 2.90)	0.80 (± 0.01) a	26.35 (± 1.47)	139.54 (± 0.49) a
BiH2-B4	36.40 (± 3.78)	0.77 (± 0.01) b	26.23 (± 1.17)	125.89 (± 0.46) bc
BiH2-B5	38.04 (± 0.67)	0.77 (± 0.01) b	24.63 (± 2.17)	124.60 (± 0.25) cd
Significance	ns	*	ns	*

*Note: SPAD value = Relative chlorophyll content measured using SPAD-502 Meter (Minolta, Japan); NDVI = Normalized Difference Vegetation Index; SD = standard deviation; CTR = control; BiH2-B1 = (10 L ha⁻¹); BiH2-B2 (12 L ha⁻¹); BiH2-B3 (15 L ha⁻¹); BiH2-B4 (20 L ha⁻¹); BiH2-B5 (25 L ha⁻¹); ns = no significant differences among treatments (one-way ANOVA); * = significant differences among treatments at the $p < 0.05$ level (one-way ANOVA); different lowercase letters indicate significant differences among treatments at the $p < 0.05$ level (Student-Newman-Keuls post-hoc test).

Table 3. Parameters measured at final assessment (27 June, BBCH 71) in post-transplant stress on *Solanum lycopersicum* L. (TR-1).

Treatment	SPAD value (SD)	NDVI (SD)	Plant Height cm (SD)	Leaf Area cm ² (SD)
CTR	32.14 (± 0.28) e	0.70 (± 0.03)	30.55 (± 2.26)	187.00 (± 0.54) d
BiH2-B1	33.50 (± 0.30) c	0.71 (± 0.01)	30.81 (± 1.96)	193.50 (± 0.04) c
BiH2-B2	35.00 (± 0.17) b	0.72 (± 0.04)	30.93 (± 4.75)	197.00 (± 0.37) b
BiH2-B3	35.46 (± 0.20) a	0.73 (± 0.03)	31.05 (± 2.91)	201.21 (± 3.80) a
BiH2-B4	33.06 (± 0.16) d	0.72 (± 0.06)	30.06 (± 1.79)	187.16 (± 0.46) d
BiH2-B5	32.48 (± 0.29) e	0.72 (± 0.07)	31.35 (± 0.61)	187.24 (± 0.97) d
Significance	*	ns	ns	*

*Note: SPAD value = Relative chlorophyll content measured using SPAD-502 Meter (Minolta, Japan); NDVI = Normalized Difference Vegetation Index; SD = standard deviation; CTR = control; BiH2-B1 = (10 L ha⁻¹); BiH2-B2 (12 L ha⁻¹); BiH2-B3 (15 L ha⁻¹); BiH2-B4 (20 L ha⁻¹); BiH2-B5 (25 L ha⁻¹); ns = no significant differences among treatments (one-way ANOVA); * = significant differences among treatments at the $p < 0.05$ level (one-way ANOVA); different lowercase letters indicate significant differences among treatments at the $p < 0.05$ level (Student-Newman-Keuls post-hoc test).

Table 4. Parameters measured only at final assessment (27 June, BBCH 71) in post-transplant stress on *Solanum lycopersicum* L. (TR-1).

Treatment	Aerial Biomass Fresh g (SD)	Aerial Biomass Dry g (SD)	Root Biomass Fresh g (SD)	Root Biomass Dry g (SD)
CTR	56.84 (± 1.36) b	10.07 (± 0.83)	29.90 (± 0.66)	3.27 (± 0.02)
BiH2-B1	59.34 (± 0.95) b	10.30 (± 0.48)	29.59 (± 0.67)	3.27 (± 0.14)
BiH2-B2	64.03 (± 7.14) a	11.77 (± 0.51)	30.52 (± 0.59)	3.30 (± 0.22)
BiH2-B3	59.05 (± 0.53) b	11.72 (± 0.48)	29.40 (± 0.49)	3.31 (± 0.01)
BiH2-B4	57.01 (± 0.63) b	10.93 (± 0.71)	29.29 (± 0.58)	3.25 (± 0.31)
BiH2-B5	56.83 (± 0.29) b	11.08 (± 0.16)	30.40 (± 0.46)	3.39 (± 0.04)
Significance	*	ns	ns	ns

*Note: SD = standard deviation; CTR = control; BiH2-B1 = (10 L ha⁻¹); BiH2-B2 (12 L ha⁻¹); BiH2-B3 (15 L ha⁻¹); BiH2-B4 (20 L ha⁻¹); BiH2-B5 (25 L ha⁻¹); ns = no significant differences among treatments (one-way ANOVA); * = significant differences among treatments at the $p < 0.05$ level (one-way ANOVA); different lowercase letters indicate significant differences among treatments at the $p < 0.05$ level (Student-Newman-Keuls post-hoc test).

3.2. Pepper trial

The physiological evaluations in the pepper trial showed that the BiH2-B treatments can mitigate cold stress symptoms (Tables 5–9), particularly when applied at 12–15 L ha⁻¹ volume. Plants treated with BiH2-B3 (15 L ha⁻¹) exhibited increased plant height (+ 7.5%), as well as higher SPAD value (+ 10.2%), along with reduced cold damage compared to the untreated control. These effects were particularly evident during the late assessments, except for the NDVI, for which higher values were reported at the third assessment (+ 8.5%). These results highlight the efficacy of the fermented broth in mitigating temperature stress in this thermophilic species. Regarding the application volumes, the observed

improvements followed a non-linear trend characterized by a plateau or a slight regression at higher doses.

Plant damage due to cold stress was visibly and significantly reduced in treated plants (Table 9), confirming the protective effect of the biostimulant. No signs of phytotoxicity such as chlorosis, necrosis, or stunted growth were observed at any rate, including the highest dose. The fermented broth showed excellent solubility and a good uniformity at the foliar spray application.

Table 5. Parameters measured at first assessment (28 June, BBCH 15) in cold stress on *Capsicum annuum* L. (TR-2).

Treatment	SPAD value (SD)	NDVI (SD)	Plant Height cm (SD)
CTR	35.03 ($\pm$ 2.41)	0.62 ($\pm$ 0.03)	29.38 ($\pm$ 0.75)
BiH2-B1	35.83 ($\pm$ 4.04)	0.63 ($\pm$ 0.06)	29.40 ($\pm$ 0.79)
BiH2-B2	36.28 ($\pm$ 0.85)	0.64 ($\pm$ 0.03)	29.58 ($\pm$ 1.09)
BiH2-B3	35.23 ($\pm$ 2.81)	0.64 ($\pm$ 0.03)	30.25 ($\pm$ 1.66)
BiH2-B4	35.45 ($\pm$ 1.32)	0.62 ($\pm$ 0.02)	28.90 ($\pm$ 0.99)
BiH2-B5	36.05 ($\pm$ 2.72)	0.63 ($\pm$ 0.02)	29.58 ($\pm$ 0.85)
Significance	ns	ns	ns

*Note: SPAD value = Relative chlorophyll content measured using SPAD-502 Meter (Minolta, Japan); NDVI = Normalized Difference Vegetation Index; SD = standard deviation; CTR = control; BiH2-B1 = (10 L ha⁻¹); BiH2-B2 (12 L ha⁻¹); BiH2-B3 (15 L ha⁻¹); BiH2-B4 (20 L ha⁻¹); BiH2-B5 (25 L ha⁻¹); ns = no significant differences among treatments (one-way ANOVA); * = significant differences among treatments at the $p < 0.05$ level (one-way ANOVA); different lowercase letters indicate significant differences among treatments at the $p < 0.05$ level (Student-Newman-Keuls post-hoc test).

Table 6. Parameters measured at second assessment (1 July, BBCH 16) in cold stress on *Capsicum annuum* L. (TR-2).

Treatment	SPAD value (SD)	NDVI (SD)	Plant Height cm (SD)
CTR	31.14 ($\pm$ 0.04)	0.35 ($\pm$ 0.04)	29.50 ($\pm$ 0.54)
BiH2-B1	31.59 ($\pm$ 1.37)	0.36 ($\pm$ 0.04)	29.63 ($\pm$ 0.91)
BiH2-B2	33.06 ($\pm$ 2.22)	0.36 ($\pm$ 0.05)	29.66 ($\pm$ 0.46)
BiH2-B3	33.69 ($\pm$ 1.64)	0.36 ($\pm$ 0.04)	30.81 ($\pm$ 1.36)
BiH2-B4	33.24 ($\pm$ 0.51)	0.35 ($\pm$ 0.02)	29.60 ($\pm$ 0.68)
BiH2-B5	33.40 ($\pm$ 1.39)	0.37 ($\pm$ 0.01)	29.84 ($\pm$ 0.63)
Significance	ns	ns	ns

*Note: SPAD value = Relative chlorophyll content measured using SPAD-502 Meter (Minolta, Japan); NDVI = Normalized Difference Vegetation Index; SD = standard deviation; CTR = control; BiH2-B1 = (10 L ha⁻¹); BiH2-B2 (12 L ha⁻¹); BiH2-B3 (15 L ha⁻¹); BiH2-B4 (20 L ha⁻¹); BiH2-B5 (25 L ha⁻¹); ns = no significant differences among treatments (one-way ANOVA); * = significant differences among treatments at the $p < 0.05$ level (one-way ANOVA); different lowercase letters indicate significant differences among treatments at the $p < 0.05$ level (Student-Newman-Keuls post-hoc test).

Table 7. Parameters measured at third assessment (3 July, BBCH 17) in cold stress on *Capsicum annuum* L. (TR-2).

Treatment	SPAD value (SD)	NDVI (SD)	Plant Height cm (SD)
CTR	29.73 ($\pm$ 4.70)	0.47 ($\pm$ 0.01) d	29.05 ($\pm$ 0.33) d
BiH2-B1	30.38 ($\pm$ 1.93)	0.47 ($\pm$ 0.01) d	29.94 ($\pm$ 0.68) c
BiH2-B2	30.64 ($\pm$ 2.57)	0.49 ($\pm$ 0.01) c	30.83 ($\pm$ 0.63) b
BiH2-B3	31.15 ($\pm$ 1.42)	0.51 ($\pm$ 0.01) a	31.90 ($\pm$ 0.71) a
BiH2-B4	31.41 ($\pm$ 1.69)	0.50 ($\pm$ 0.01) b	31.90 ($\pm$ 0.39) a
BiH2-B5	31.00 ($\pm$ 4.25)	0.49 ($\pm$ 0.01) c	32.23 ($\pm$ 0.37) a
Significance	ns	*	*

*Note: SPAD value = Relative chlorophyll content measured using SPAD-502 Meter (Minolta, Japan); NDVI = Normalized Difference Vegetation Index; SD = standard deviation; CTR = control; BiH2-B1 = (10 L ha⁻¹); BiH2-B2 (12 L ha⁻¹); BiH2-B3 (15 L ha⁻¹); BiH2-B4 (20 L ha⁻¹); BiH2-B5 (25 L ha⁻¹); ns = no significant differences among treatments (one-way ANOVA); * = significant differences among treatments at the $p < 0.05$ level (one-way ANOVA); different lowercase letters indicate significant differences among treatments at the $p < 0.05$ level (Student-Newman-Keuls post-hoc test).

Table 8. Parameters measured at fourth assessment (6 July, BBCH 18) in cold stress on *Capsicum annuum* L. (TR-2).

Treatment	SPAD value (SD)	NDVI (SD)	Plant Height cm (SD)
CTR	32.28 ($\pm$ 0.48) d	0.68 ($\pm$ 0.02)	32.45 ($\pm$ 0.39) d
BiH2-B1	36.35 ($\pm$ 0.27) b	0.68 ($\pm$ 0.04)	32.55 ($\pm$ 0.39) d
BiH2-B2	36.41 ($\pm$ 0.30) b	0.69 ($\pm$ 0.01)	33.63 ($\pm$ 0.59) c
BiH2-B3	36.41 ($\pm$ 0.41) b	0.69 ($\pm$ 0.01)	34.81 ($\pm$ 0.21) b
BiH2-B4	37.65 ($\pm$ 0.13) a	0.68 ($\pm$ 0.01)	35.48 ($\pm$ 0.25) a
BiH2-B5	34.83 ($\pm$ 0.50) c	0.68 ($\pm$ 0.01)	35.59 ($\pm$ 0.42) a
Significance	*	ns	*

*Note: SPAD value = Relative chlorophyll content measured using SPAD-502 Meter (Minolta, Japan); NDVI = Normalized Difference Vegetation Index; SD = standard deviation; CTR = control; BiH2-B1 = (10 L ha⁻¹); BiH2-B2 (12 L ha⁻¹); BiH2-B3 (15 L ha⁻¹); BiH2-B4 (20 L ha⁻¹); BiH2-B5 (25 L ha⁻¹); ns = no significant differences among treatments (one-way ANOVA); * = significant differences among treatments at the $p < 0.05$ level (one-way ANOVA); different lowercase letters indicate significant differences among treatments at the $p < 0.05$ level (Student-Newman-Keuls post-hoc test).

Table 9. Parameters measured at final assessment (16 July, BBCH 44) in cold stress on *Capsicum annuum* L. (TR-2).

Treatment	SPAD value (SD)	NDVI(SD)	Height cm (SD)	Tissue damage (SD)†
CTR	39.23 ($\pm$ 0.20) d	0.82 ($\pm$ 0.01)	37.73 ($\pm$ 0.42) c	2.50 ($\pm$ 0.58) a
BiH2-B1	39.95 ($\pm$ 0.37) c	0.83 ($\pm$ 0.01)	37.98 ($\pm$ 0.31) c	0.00b
BiH2-B2	43.21 ($\pm$ 0.37) a	0.83 ($\pm$ 0.01)	38.36 ($\pm$ 0.51) c	0.00b
BiH2-B3	43.25 ($\pm$ 0.34) a	0.84 ($\pm$ 0.04)	40.55 ($\pm$ 0.29) a	0.00b
BiH2-B4	42.18 ($\pm$ 0.28) b	0.82 ($\pm$ 0.01)	40.28 ($\pm$ 0.42) ab	0.00b
BiH2-B5	41.84 ($\pm$ 0.39) b	0.82 ($\pm$ 0.01)	39.73 ($\pm$ 0.55) b	0.00b
Significance	*	ns	*	*

*Note: SPAD value = Relative chlorophyll content measured using SPAD-502 Meter (Minolta, Japan); NDVI = Normalized Difference Vegetation Index; SD = standard deviation; CTR = control; BiH2-B1 = (10 L ha⁻¹); BiH2-B2 (12 L ha⁻¹); BiH2-B3 (15 L ha⁻¹); BiH2-B4 (20 L ha⁻¹); BiH2-B5 (25 L ha⁻¹); ns = no significant differences among treatments (one-way ANOVA); * = significant differences among treatments at the $p < 0.05$ level (one-way ANOVA); different lowercase letters indicate significant differences among treatments at the $p < 0.05$ level (Student-Newman-Keuls post-hoc test); † = Tissue damage calculated based on a visual scoring system.

4. Discussion

The climatic-chamber trials on tomatoes (TR-1) and peppers (TR-2) indicate that application of the fermentation-derived biostimulant BiH2-B alleviates abiotic stresses in these two thermophilic horticultural crops, primarily by sustaining chlorophyll-related parameters and growth dynamics under challenging conditions. In tomatoes, where the trial was designed to exacerbate post-transplant stress and root establishment, BiH2-B improved the canopy performance across the vegetative cycle: SPAD and leaf area increased with doses up to 15 L ha⁻¹, and this was accompanied by higher aerial fresh biomass (maximized at intermediate volume around 12 L ha⁻¹) and an overall enhancement of vegetative growth compared with the untreated control, while the highest doses (20–25 L ha⁻¹) largely maintained, rather than amplified, these responses, and did not induce phytotoxicity. In peppers, when exposed to an acute chilling event (6 °C for 48 h), BiH2-B-treated plants showed significantly higher NDVI values at the third assessment (BBCH 17) with an application volume of 15 L ha⁻¹; significantly higher SPAD and plant height values were also reported with an application volume of 15 L ha⁻¹, in particular at the final assessment. Furthermore, the BiH2-B application significantly reduced cold-induced damage during the recovery phase at all application volumes tested (10–25 L ha⁻¹), without any symptoms of chlorosis, necrosis, or growth inhibition. Higher rates of BiH2-B treatment only gave significant improvements in the late measurements, which are likely related to the improve root absorption capacity. This shared, non-linear dose-response is typical of plant biostimulants, which often present an optimal range in which the regulatory effect is effectively expressed [13,29].

Although the present study was not designed to investigate molecular mechanisms, the observed physiological responses allow some hypothesis-driven interpretation. The improvements in SPAD, NDVI, canopy development, and stress recovery are consistent with the known effects of low-molecular-weight compounds, including amino acids, oligopeptides, and organic acids, on nitrogen assimilation, photosynthetic efficiency, and stress-responsive metabolic pathways. These compounds are widely reported to act as metabolic precursors and signaling molecules, thereby enhancing plant

resilience under abiotic stress without exerting a direct nutritional effect [30]. Therefore, the biostimulant activity of BiH2-B is likely attributable to a regulatory rather than a fertilizing role, which is in line with the EU definition of plant biostimulants [8].

The observed increases in SPAD and NDVI in both species are consistent with previous work in horticultural crops, where different classes of biostimulants improved the chlorophyll content, photosynthetic activity, and biomass accumulation under suboptimal or stressful growing [31–33]. Across both species, the consistent improvement of SPAD, NDVI, and canopy development under stress conditions indicates an enhanced physiological resilience rather than a generalized growth stimulation, thus supporting a regulatory rather than a nutritional mode of action. Likewise, the mitigation of chilling damage in peppers aligns with studies showing that foliar fermentation-based biostimulants or regulatory molecules (e.g., cyanobacterial extracts, melatonin, humic substances) can enhance SPAD, antioxidant capacity, and stress tolerance in *Capsicum* spp. subjected to abiotic stresses such as salinity, low temperatures, or drought [17,34]. The coherent responses observed in tomatoes and peppers suggest that BiH2-B exerts a multi-target action on plant functioning, promoting a faster restoration of the photosynthetic capacity and canopy expansion after stress episodes and potentially stimulating root activity and resource acquisition, as reported for other hydrolyzed protein, humic or microbial-based formulations [14,17,29]. The ability of amino acids and protein hydrolysates such as those contained in BiH2-B to stimulate primary plant metabolism and to enhance nitrogen uptake and assimilation has been documented by several authors [15,34]. In addition, numerous studies have shown that protein hydrolysates and amino acids can induce secondary metabolism and strengthen plant defense responses against abiotic stresses [33,16]. Importantly, BiH2-B is obtained from exhausted fermentation broth of a dark fermentation process, a type of biorefinery co-product that is increasingly recognized as a promising source of bioactive compound such as oligopeptides, organic acids, and amino acids with biostimulant activity, and is a strong fit within circular-bioeconomy strategies [19]. The present evidence that such a fermentation-derived product can improve physiological resilience to transplant-related stress in tomatoes and chilling stress in peppers supports its positioning as an effective, safe, and resource-efficient biostimulant for high-value horticultural crops, which is in line with the current emphasis of the biostimulant literature on climate-resilient and environmentally sustainable production systems.

5. Conclusions

The climatic-chamber experiments on tomatoes and peppers demonstrated that the fermentation-derived biostimulant BiH2-B can effectively mitigate abiotic stress in thermophilic horticultural crops, particularly when applied at moderate rates. In both trials, clear and consistent physiological responses were observed at intermediate doses (12–15 L ha⁻¹), as reflected by increased SPAD values, improved canopy development, and biomass accumulation, and reduced tissue damage under stress conditions. These findings indicate that the efficiency of BiH2-B is rate dependent, with an optimal application window beyond which further dose increases do not translate into additional agronomic benefits and may be less resource-efficient. The absence of phytotoxic symptoms across all treatments, together with the ease of handling and application, supports the suitability of this product for practical use in commercial horticultural systems. Overall, the present results position BiH2-B as a promising, safe, and circular-bioeconomy-compatible biostimulant to enhance crop resilience to transplant-related and chilling stress in high-value vegetable crops. Future research should include molecular and biochemical investigations to further elucidate the mechanisms underlying the observed stress mitigation effects, which were beyond the scope of the present physiological study.

Such studies will be essential to validate the efficacy of BiH2-B under commercial conditions, refine dose–response recommendations, and assess its integration into broader climate-resilient and sustainable crop production strategies.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflicts of interest

The authors declare no conflicts of interest. The funder had no role in the design of the experiments; in the collection, analysis, or interpretation of data; or in the decision to publish the results.

Data availability statement

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

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