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Root Zone Temperature Determines the Biostimulant Effect of Humic Acids on Nutrient Acquisition in Cucumber Seedlings

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Abstract
Humic acid–based biostimulants are increasingly used in plant production due to their ability to influence plant performance. However, their effectiveness is highly variable and strongly dependent on environmental conditions. Root zone temperature represents a key regulator of seedling quality, influencing growth patterns and nutrient acquisition. To clarify the environment-dependent mode of action of humic acids, this study investigated their effects on the morphology, physiology and mineral composition of cucumber (*Cucumis sativus* L. 'Sargo') seedlings grown under different root zone temperatures and irrigation timings. Root zone temperature emerged as the primary driver of seedling performance. Warmer conditions enhanced shoot growth, increasing shoot dry mass by 10% and leaf area and plant height by approximately 30%, while suppressing root development. In contrast, cooler root zone conditions promoted root growth, increasing root dry mass by 10% and improving resource-use efficiency by more than 10%. Application of the humic acid biostimulant did not significantly affect seedling morphology or physiological traits but exerted a clear temperature-dependent effect on nutrient accumulation, enhancing mineral nutrient concentrations predominantly under cooler root zone conditions. These findings demonstrate that humic acid–based biostimulants primarily regulate nutrient acquisition rather than visible growth responses and that their efficacy is strongly governed by root zone temperature, highlighting the importance of environmental context in biostimulant application strategies.

Keywords Irrigation · Morphology · Physiology · Quality · Water use efficiency · Nitrogen use efficiency · Shoot

Introduction

Most species in the Cucurbitaceae family are commercially grown by seedling transplants (Bantis and Koukounaras 2023). This practice improves plant uniformity, optimises spacing, reduces weed pressure, and enables earlier and higher yields (Gallegos-Cedillo et al. 2024a, b; Leskovar and Othman 2021). Nevertheless, cucumbers are still partly established by direct seeding, primarily due to their rapid early growth, with seedlings reaching transplantable size within 1.5–3 weeks (Gallegos-Cedillo et al. 2024a, b), allowing effective weed competition and lower production costs (Vavrina 2002). A major limitation of cucumber transplanting is transplant shock, caused by mechanical disturbance, root damage, and abrupt exposure to field conditions. This often results in a temporary reduction in shoot growth, due to sudden changes in growth conditions and resource use restrictions (Agehara 2020; Kerbiriou et al. 2013; Li et al. 2016; Grossnickle 2005). Although young plants generally possess high regenerative capacity (Kunc et al. 2024; Sun and Zhu 2021), resulting in minimal long-term effects on their performance (Gavrić and Omerbegović 2021; Leskovar and Othman 2021), the use of actively growing, high quality seedlings remains essential for rapid recovery and successful field establishment (Bantis et al. 2019; Bantis and Koukounaras 2023; Jeong et al. 2024; Ronga et al. 2021). Optimizing seedling quality is therefore essential to overcome establishment constraints and fully utilize the agronomic advantages of cucumber transplanting.

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Seedling quality is determined by a combination of morphological, nutritional, and physiological traits. Morphological characteristics such as leaf area, shoot and root development, stem thickness, and plant height are widely used indicators of seedling vigour. A well-developed root system and balanced shoot to root relationship enhance tolerance to transplant-related stress (Binotto et al. 2010; Mašková and Herben 2018; Yan et al. 2023). In addition to morphology, nutritional status is also a key quality trait, as fertilisation practices in nurseries aim to build nutrient reserves that can be rapidly remobilised to support early shoot and root growth after transplanting (Grossnickle and MacDonald 2018). Adequate physiological performance is also required to meet the energy demands of active growth (Chauhan et al. 2023). Cucurbitaceae exhibit one of the least favourable root to shoot ratios of all vegetables (Qin and Leskovar 2020), highlighting the need for improved cultivation techniques.

To reach desired seedling quality, several factors are carefully managed. One of the most important components is temperature (Gallegos-Cedillo et al. 2024a, b). An effective strategy to create suitable temperature conditions for seedlings is to directly heat the root zone area. By directly heating the plant where heat is needed, we can avoid overheating the air and stressing the shoots while keeping roots active. This method has been shown to improve the uniformity and overall quality of seedlings, particularly during colder seasons (He et al. 2020).

Irrigation timing can also alter the soil temperature (Ismail et al. 2007). This can be important, especially for plants like cucumbers that prefer higher temperatures. Cleverly scheduled irrigation can also achieve early root development (Franco and Leskovar 2002). Moreover, well managed irrigation practices can reduce water use while simultaneously promoting desirable traits (Adams and Zeleke 2017).

One of additional improvements of seedling cultivation techniques can be the use of biostimulants. Among these, humic substances have received increasing attention for their potential to enhance seedling quality (Qin and Leskovar 2020). Their hormone-like activity, enabled by functional groups within humic molecules, can stimulate plasma membrane H^+-ATPase activity and enzymes involved in nutrient uptake, while also promoting root growth and development (De Pascale et al. 2017; Yang et al. 2021; Faria de Souza et al., 2025; Nardi et al. 2021; Mora et al. 2010). Through these mechanisms, humic substances may enhance seedling quality parameters previously discussed and provide benefits even in controlled plant production systems, such as nurseries (Gallegos-Cedillo et al. 2024a, b). However, these outcomes are known to be sensitive to root zone temperature (Bassirirad 2000; Zhang et al. 2006), suggesting that the biostimulant effectiveness of humic substances may depend on thermal conditions.

This study aimed to evaluate the effects of a humic acid–based biostimulant on cucumber seedling quality under different root zone temperatures and irrigation timing conditions. Specifically, we assessed whether biostimulant application influences seedling morphology, physiology, and nutrient composition, and whether these responses are modulated by root zone temperature. This work seeks to elucidate the environment-dependent mode of action of humic acid–based biostimulants and their potential role in optimizing cucumber seedling production.

Materials and Methods

A greenhouse experiment was conducted at the Biotechnical Faculty, University of Ljubljana, Slovenia (46°2’55"N latitude, 14°28’22"E longitude, 295 m altitude) from March 4 to April 5, 2024. The study focused on cucumber seedlings (*Cucumis sativus* L. ‘Sargo’). The seeds used in the experiment were obtained from Austrosaat (Wien, Austria). Seeds were sown on March 3 into 48 6-cell seed trays (288 plants) with Neuhaus N3 substrate and germinated in a growth chamber at 23 °C and 95% relative humidity until the emergence of the first sprouts on March 8. Once the majority of seeds had germinated, seedlings were fully irrigated and transferred onto propagation units X-Stream (Nutriculture UK Ltd., Skelmersdale, UK) under two temperature regimes: first group were plants with root zone heated on 28 °C (designated as WARM; actual temperatures 20–30 °C), others remained unheated (designated as COOL; actual temperatures 10–25 °C). Root zone temperature was selected by Gosselin and Trudel (1985) as optimal for cucumber shoot development.

To half of the plants in each root zone temperature regime, 10 mL of the biostimulant Humistar® (Tradecorp, Rovensa, Madrid, Spain) was applied per plant at a concentration of 0.02% (designated as H). The remaining plants served as a control group and received 10 mL of water instead (designated as C). The biostimulant was applied twice, once at the appearance of the first true leaf and again 10 days later. According to the manufacturer, Humistar® contains 13.2% humic acids, 16.5% total humic extract, 5.5% K_2O, and 3.3% fulvic acids (w/w %), with a pH of 13.

Each combination of root zone temperature and biostimulant treatment was further divided into three irrigation timing groups. First was watered at approximately 9 o’clock am (designated as am), second was watered at approximately 1 o’clock pm (designated as noon), and the

last was watered at approximately 7 o'clock pm (designated as pm). Water used for irrigation had a temperature of 11 ± 0.2 °C. To ensure uniform saturation, irrigation was performed by dipping the seedling trays into water until the substrate was fully soaked.

For each treatment combination, four seedling trays were sown, providing a total of 24 plants per treatment. From these, 10 plants were selected for detailed analysis. Seedlings were fertigated twice with a 150 ppm solution of a balanced 20-20-20 NPK + TE fertilizer (Lima Europe NV, Rumst, Belgium). The fertilizer composition was as follows: 5.8% nitrate nitrogen (N-NO_3), 3.8% ammonium nitrogen (N-NH_4), 10.4% urea nitrogen (N-NH_2), 20% phosphorus (P_2O_5), and 20% potassium (K_2O). It also contained the following trace elements: 0.01% boron (B), 0.003% copper (Cu), 0.025% iron (Fe), 0.013% manganese (Mn), 0.0018% molybdenum (Mo), and 0.004% zinc (Zn).

Throughout the experiment, temperature was recorded for each combination of root zone temperature and irrigation timing, within the substrate and just above it at plant level. A diurnal course of root zone temperature based on heating and irrigation level for a chosen irrigation day is presented in Fig. 1.

For root zone temperature we used T-Soil sensors (Ecomatik, Dachau, Germany) connected to data loggers DL18 BLE Data Logger (Ecomatik, Dachau, Germany) for soil temperature and Voltcraft DL-121TH Data Logger (Voltcraft, Germany) for air temperature. In addition, ambient light radiation was monitored in an adjacent open field. Air temperature and light conditions during the growth period are presented in Fig. 2. In greenhouse no additional light was provided besides the sun radiation and ventilation was set to maintain 23 °C.

Morphological Measurements

To assess seedling quality in terms of morphology, plants were sampled at the point of fully developed seedlings at stage of two fully developed leaves (Vavrina 2002). The roots of the cucumber seedlings were carefully washed to remove any remaining substrate. Following this, seedling height (H) (measured from the base to the top of the shoot) and stem diameter (SD) at the root collar were recorded. The relative chlorophyll content (SPAD) was then measured using a Minolta SPAD-502 chlorophyll meter (Tokyo, Japan). SPAD was expressed as the mean of three measurements on the first fully expanded leaf. Next, the shoot (SFM) and root (RFM) fresh mass was determined using a precision scale (Kern ALS 120–4 N, Albstadt, Germany) and leaf area was assessed using the LI-3000 portable leaf area meter (Licor, Lincoln, Nebraska, USA). To determine shoot and root dry mass (SDM, RDM), the fresh samples were placed in a drying oven at 50 °C until they reached a constant mass.

From the assessed data, Dixon Quality Index (DQI) (1), Compactness (2), and Root to Shoot Ratio (RS ratio) (3) were calculated (Jang et al. 2020).

$$DQI = \frac{Total\ dry\ weight\ (Shoot + Root)}{\frac{Plant\ height}{Stem\ diameter} + \frac{Shoot\ dry\ weight}{Root\ dry\ weight}} \quad (1)$$

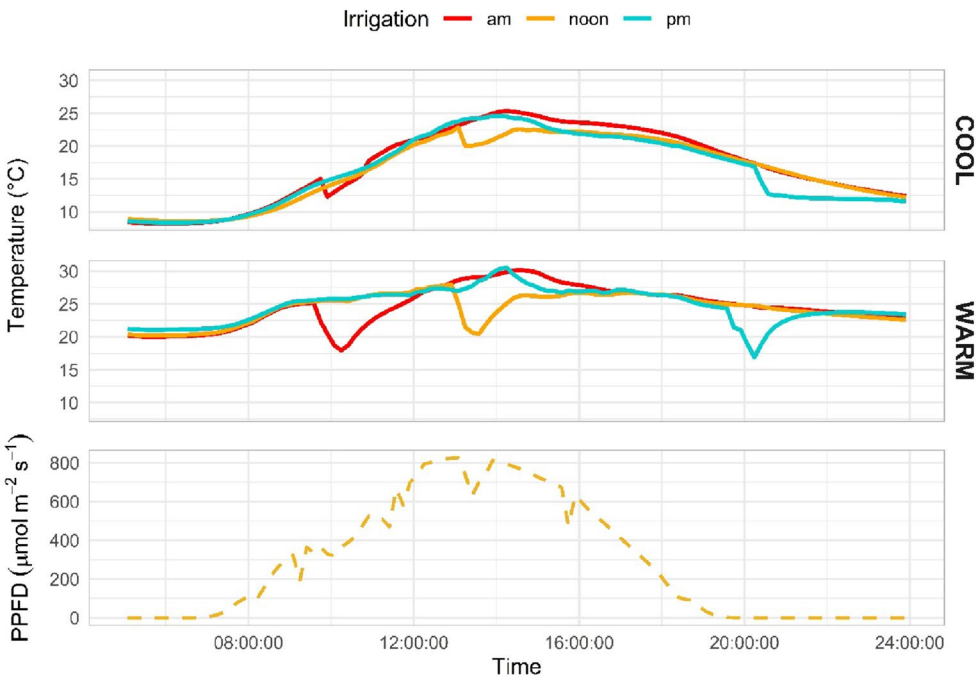


Fig. 1 Daily course of root zone temperature under three irrigation timings (red - am, orange -noon and cyan - pm), shown separately for each root zone temperature and photosynthetic photon flux density (PPFD) measured throughout the day

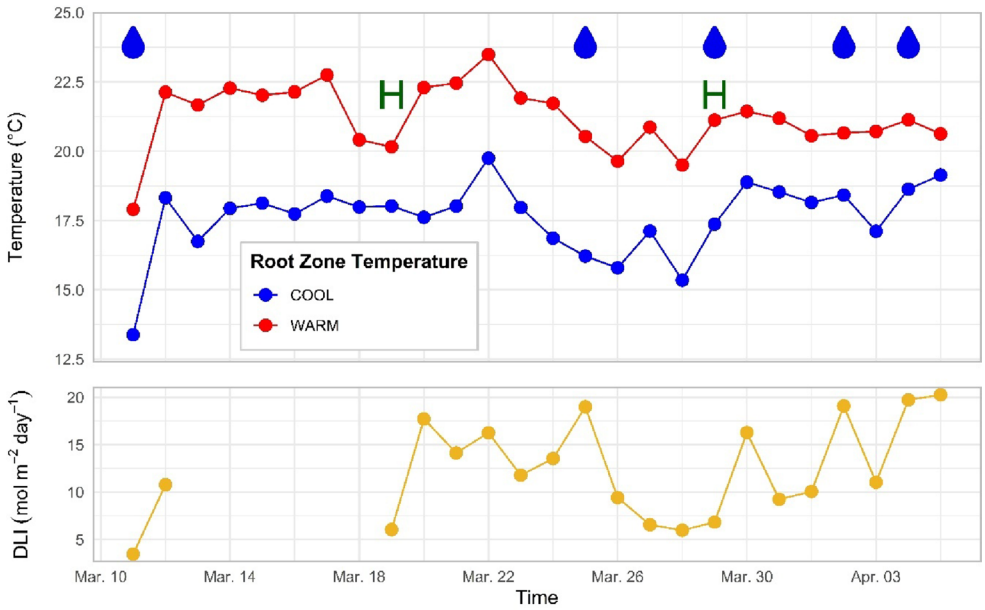


Fig. 2 Average daily temperature above the heated (red - WARM) and unheated substrate (blue – COOL) and daily light integral (DLI) throughout the experiment. Water droplet symbols on the top indicate irrigation days and H marks the days when the biostimulant was applied

$$Compactness\ (g/cm) = \frac{Shoot\ dry\ weight}{Plant\ height} \quad (2)$$

$$RS\ ratio = \frac{Root\ dry\ weight}{Shoot\ dry\ weight} \quad (3)$$

Physiology Measurements

Physiological parameters were measured using the CI-340 Handheld Photosynthesis System (CID Bio-Science, Camas, Washington, USA). Measurements were taken on the second fully expanded leaf of fully irrigated cucumber plants under natural sunlight conditions in a greenhouse. The following gas exchange parameters were recorded: Net photosynthesis rate (A), Stomatal conductance (gs), Transpiration rate (E) and Intercellular CO_2 concentration (Ci).

To account for diurnal variation in plant physiological responses, a randomised complete block design was implemented. One measurement per treatment was taken sequentially per block, with a total of 13 blocks assessed over two days, from 8:00 to 15:00 on the first day and from 8:00 to 10:00 on the second day. Environmental conditions in the measurement chamber varied throughout the day and between blocks. Photosynthetically active radiation (PAR) ranged from 100 to 200 µmol m^{-2} s^{-1}, with three blocks showing higher light intensity (~ 500 µmol m^{-2} s^{-1}). Air temperature increased gradually from approximately 20 °C in the morning to 30 °C in the afternoon. Correspondingly, vapor pressure deficit (VPD) rose from 0.7 up to 2.3 kPa. The greenhouse was ventilated during the measurement period to avoid CO_2 build up. Ambient CO_2 concentration ranged between 500 and 600 ppm.

Mineral Composition

For each observation, aboveground plant material from ten individual plants was collected, oven-dried at 50 °C to constant mass, and subsequently ground to a fine powder used for nutrient analysis. Mineral composition analysis focused on macronutrients and selected micronutrients, of which iron was included due to its central role in cucumber nutrition and its known responsiveness to humic substances (Zuluaga et al. 2023), while sodium was used as an indicator element reflecting changes in ion balance (Shalaby & El-Messairy, 2018). Total nitrogen (N_{total}) content was analysed at the Chair of Soil and Environmental Science, Agronomy Department, University of Ljubljana (Slovenia), according to SIST ISO 13878:1999. Concentrations of macro elements phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), and sulfur (S), as well as the micronutrients iron (Fe) and sodium (Na) were determined at Bureau Veritas Commodities Canada Ltd. (Vancouver, Canada) using their VG101 method, involving acid digestion with HNO_3, followed by aqua regia and subsequent analysis by inductively coupled plasma mass spectrometry (ICP-MS).

Water and Nitrogen Use Efficiency

Additionally, water use efficiency (WUE) and nitrogen use efficiency (NUE) were calculated for each treatment. WUE was expressed as the ratio of average plant dry mass to the average amount of water used to produce

Table 1 Morphological traits component loadings. SPAD, H – height, SD-stem diameter, SFM- shoot fresh mass, RFM-root fresh mass, LA – leaf area, SDM-shoot dry mass, RDM-root dry mass

	PC1	PC2	PC3
SPAD	−0.16143372	−0.073261043	**−0.95207643**
H	**0.39085470**	**0.313399012**	0.04126441
SD	**0.29814763**	−0.090499874	−0.18883972
SFM	**0.50185714**	0.025724939	−0.15368159
RFM	0.14297975	**−0.654117296**	0.10471969
LA	**0.47192078**	0.049014354	0.02574690
SDM	**0.48172937**	−0.002228228	−0.13825687
RDM	0.07213561	**−0.676228564**	0.04272215

that biomass. To determine total water use, seedling trays were weighed before and after each irrigation event throughout the experiment. The cumulative water input per treatment was then divided by the number of plants to obtain the average water use per plant. WUE was expressed as grams of dry matter produced per millimetre of water used (g DM mm^{-1} H_2O). NUE was calculated as the ratio of average plant dry mass to the amount of nitrogen content in that biomass and expressed as grams of dry matter produced per gram of nitrogen (g DM g^{-1} N) (Kerbiriou et al. 2013).

Statistical Analysis

The experiment followed a three-factor factorial design, arranged in a completely randomized layout. The three factors included root zone temperature (levels: WARM and COOL), biostimulant application (levels: H and C), and irrigation timing (levels: am, noon, and pm). This resulted in a total of 12 treatment combinations (2 × 2 × 3).

To assess whether experimental factors influenced the overall structure of seedling quality, we evaluated three distinct aspects of seedling traits: (1) morphological traits, (2) physiological traits, and (3) mineral composition. To reduce dimensionality and minimize the risk of type I error associated with multiple univariate comparisons, we conducted Principal Component Analysis (PCA) on each trait group. Subsequent analyses were limited to the first two or three principal components, cumulatively explaining at least 80% of the total variance. Analyses were conducted in the R statistical environment (R Core Team 2025) using packages including dplyr, ggplot2, factoextra, psych, lme4, and multcomp.

The effects of root zone temperature (T), biostimulant application (BS), and irrigation timing (Irr), and their two-way and three-way interactions, on the PCA scores were tested using analysis of variance (ANOVA) for morphological traits. In the case of physiological traits, linear mixed-effects models were used to account for diurnal variation as a random factor (lmer function, lme4 package). Due to limited replication, only T, BS, and their interaction were tested for their effect on mineral composition. These were evaluated using two-way ANOVA on PCA scores. The same goes for WUE and NUE analysis. Means and standard errors of examined variables are presented in Tables 2, 4 and 6. Where significant treatment effects were found, post-hoc comparisons were performed using custom contrasts (glht, from the multcomp package).

For visualisation of the results, the factoextra and ggplot2 packages were used. We reversed and renamed some principal component values, if necessary, to get a more intuitive visualization of results.

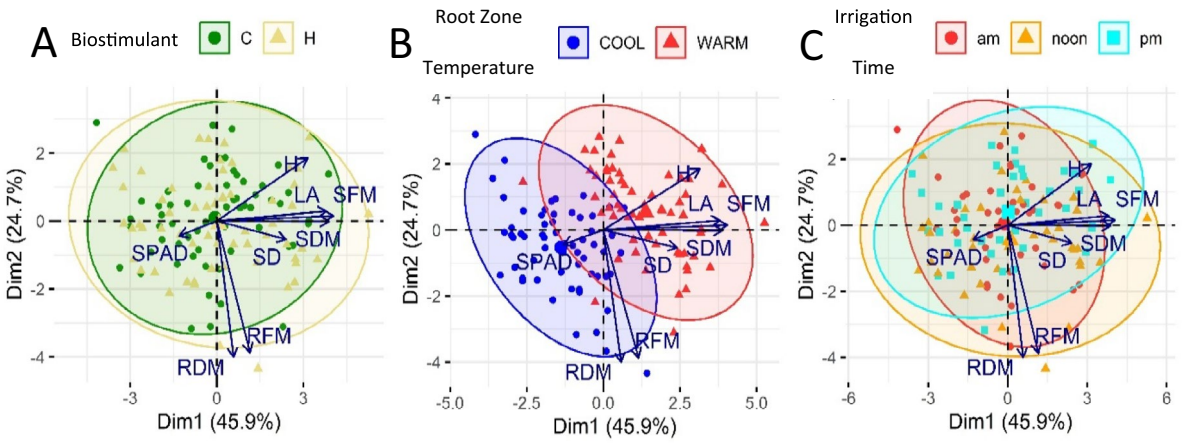


Fig. 3 Principal Component Analysis (PCA) biplot of morphological traits. Each biplot displays PC1 and PC2, which together explain 71% of the total variance. Arrows indicate the loadings of morphological traits. Colours represent groups based on one of three experimental factors, with 95% confidence ellipses drawn around group centroids to illustrate separation in morpho-space. Biplot A shows grouping by levels of biostimulant, B by root zone temperature, and C by irrigation timing

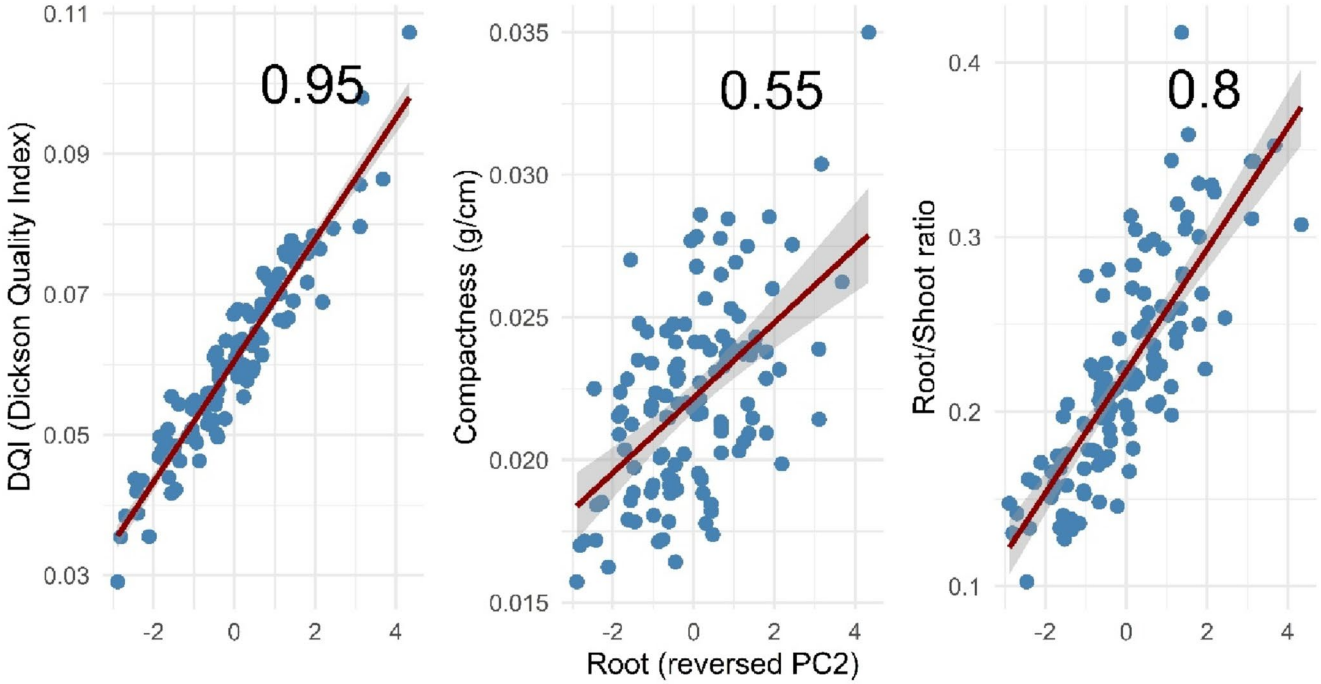


Fig. 4 Scatterplots showing the relationships between Root (reversed PC2 scores) and seedling quality traits (from left to right Dickson Quality Index (DQI), Compactness, and Root/Shoot ratio). Each panel includes a fitted linear regression line with a 95% confidence interval (shaded gray area) and the Pearson correlation coefficient is displayed in the top right corner of each plot

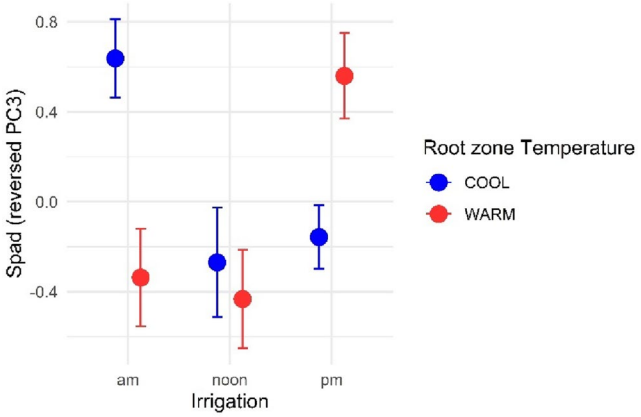


Fig. 5 Mean Spad values (reversed PC3)±standard errors across irrigation treatments for different root zone temperatures. Colours distinguish between root zone temperature conditions (blue for COOL and firebrick red for WARM)

Results

Morphological Traits

For the morphological analysis, 3 principal components (PCs) were analysed. First explains 46% of variance, second 25%, and third 12%, so together 83% of data variance is explained.

PC1 primarily reflects shoot biomass and size, with high contributions from SFM, SDM, LA, and H. Meanwhile PC2 inversely represents root biomass, with strong negative associations to RFM and RDM. Thus, lower PC2 values indicate larger root systems. The third PC is dominated by SPAD, which is strongly negatively loaded, suggesting that it reflects leaf greenness, with higher PC3 values indicating lower SPAD measurements. Correlations between original variables and each PC are shown in Table 1.

Figure 3 illustrates the influence of the experimental factors on aboveground and root traits of cucumber seedlings. In the case of the biostimulant treatment (Fig. 3A), the morphological characteristics largely overlapped with the control, indicating that biostimulant application did not affect shoot or root traits under the tested conditions.

The effect of irrigation timing (Fig. 3C) also showed considerable overlap among treatments, although slight shifts in the ellipses suggest minor variations. In contrast, a more distinct separation was observed between the two root zone temperature levels (Fig. 3B). Seedlings grown in the unheated conditions developed smaller aboveground parts (lower PC1 values), while those grown in the warm conditions produced more vigorous shoot growth. Conversely, root development was enhanced in the cooler temperature, as reflected by lower PC2 values (indicating greater root mass), whereas the warm temperature suppressed root traits but promoted shoot development.

These patterns were confirmed by ANOVA. High root zone temperature significantly reduced root traits, while

Table 2 Morphological parameters of cucumber seedlings grown under two root zone temperature conditions (WARM and COOL), treated with either biostimulant (H) or control (C), across three irrigation timings: morning (am), midday (noon), and evening (pm). Data are presented as mean ± standard deviation ($n = 10$) for the following traits: SPAD chlorophyll index, plant height (H), stem diameter (SD), root fresh mass (RFM), root dry mass (RDM), shoot fresh mass (SFM), shoot dry mass (SDM), leaf area (LA), and a whole plant dry mass (PDM). Additionally, quality indices are included: root to shoot ratio (RS), Dickson quality index (DQI), and compactness (Comp)

T	BS	Irr	SPAD	H (cm)	SD (mm)	RFM (g)	SFM (g)	LA (cm^2)	SDM (g)	RDM (g)	RS	DQI	Comp (g/cm)	PDM (g)
COOL	**H**	**am**	36.5 ± 0.52	18.1 ± 0.52	4.65 ± 0.13	1.90 ± 0.15	6.06 ± 0.19	109.0 ± 3.56	0.400 ± 0.021	0.090 ± 0.007	0.225 ± 0.015	0.0592 ± 0.0052	0.0223 ± 0.0014	0.525 ± 0.013
		noon	37.4 ± 0.51	17.8 ± 0.56	4.51 ± 0.11	2.06 ± 0.16	6.40 ± 0.15	112.0 ± 3.87	0.423 ± 0.011	0.103 ± 0.008	0.245 ± 0.019	0.0648 ± 0.004	0.0239 ± 0.0008	0.490 ± 0.029
		pm	34.1 ± 0.47	16.2 ± 0.62	4.57 ± 0.08	2.05 ± 0.11	5.77 ± 0.20	103.0 ± 3.10	0.378 ± 0.012	0.106 ± 0.006	0.284 ± 0.021	0.0682 ± 0.003	0.0235 ± 0.0009	0.474 ± 0.021
	C	**am**	36.0 ± 0.94	16.4 ± 0.42	4.62 ± 0.08	2.17 ± 0.17	5.88 ± 0.20	96.8 ± 6.17	0.384 ± 0.022	0.106 ± 0.009	0.277 ± 0.019	0.0683 ± 0.006	0.0236 ± 0.0017	0.490 ± 0.026
		noon	34.7 ± 0.50	16.4 ± 0.73	4.68 ± 0.07	2.02 ± 0.09	6.04 ± 0.26	103.0 ± 2.98	0.400 ± 0.023	0.0999 ± 0.007	0.257 ± 0.021	0.0667 ± 0.004	0.0246 ± 0.0014	0.484 ± 0.014
		pm	35.4 ± 0.53	18.4 ± 0.59	4.66 ± 0.06	1.98 ± 0.14	5.98 ± 0.21	98.1 ± 5.66	0.379 ± 0.017	0.0948 ± 0.006	0.251 ± 0.013	0.0598 ± 0.003	0.0207 ± 0.0007	0.500 ± 0.023
WARM	**H**	**am**	34.0 ± 0.68	22.8 ± 0.57	4.49 ± 0.06	1.90 ± 0.13	7.14 ± 0.13	122.0 ± 1.85	0.464 ± 0.011	0.0941 ± 0.007	0.204 ± 0.018	0.0554 ± 0.003	0.0205 ± 0.0008	0.560 ± 0.030
		noon	34.0 ± 0.82	22.2 ± 0.81	4.83 ± 0.12	1.98 ± 0.17	7.12 ± 0.22	125.0 ± 3.48	0.467 ± 0.024	0.0933 ± 0.009	0.199 ± 0.014	0.0578 ± 0.004	0.0211 ± 0.0008	0.617 ± 0.025
		pm	33.1 ± 0.50	23.0 ± 0.59	4.74 ± 0.08	2.01 ± 0.17	7.32 ± 0.17	129.0 ± 4.27	0.479 ± 0.014	0.0935 ± 0.009	0.193 ± 0.016	0.0570 ± 0.004	0.0210 ± 0.0008	0.570 ± 0.017
	C	**am**	33.7 ± 0.83	23.8 ± 0.44	4.92 ± 0.07	2.28 ± 0.09	7.99 ± 0.22	138.0 ± 4.84	0.512 ± 0.024	0.106 ± 0.004	0.210 ± 0.011	0.0634 ± 0.002	0.0215 ± 0.0009	0.558 ± 0.011
		noon	35.8 ± 0.55	22.0 ± 0.63	4.99 ± 0.11	1.67 ± 0.08	7.79 ± 0.24	140.0 ± 4.74	0.504 ± 0.019	0.0818 ± 0.004	0.163 ± 0.007	0.0553 ± 0.002	0.0229 ± 0.0006	0.572 ± 0.021
		pm	35.3 ± 0.69	23.6 ± 0.58	4.58 ± 0.13	1.60 ± 0.12	7.29 ± 0.17	136.0 ± 5.83	0.488 ± 0.018	0.0815 ± 0.009	0.169 ± 0.011	0.0508 ± 0.003	0.0208 ± 0.0009	0.586 ± 0.021

Table 3 Principal component loadings of physiological traits. Net photosynthesis rate (A), stomatal conductance (gs), transpiration rate (E) and intercellular CO_2 concentration (Ci)

	PC1	PC2
A	0.6014660	−0.1519701
E	0.5946719	−0.2436644
GS	**−0.0270644**	−0.8706891
Ci	−0.5327960	0.3992909

increasing aboveground growth. However, the effect of temperature on shoot development varied with irrigation timing. For example, shoot growth was somewhat suppressed under morning irrigation compared to noon irrigation when root zone was heated. Root development was also affected by irrigation timing, being less pronounced in the evening irrigation than at noon. The biostimulant treatment, as well as its two-way and three-way interactions with the other factors, had no significant effect on shoot or root morphological traits.

The correlation of reversed PC2 score named Root and major seedling quality parameters, calculated from the same data as principal components, are shown in Fig. 4. High correlation is between root traits and DQI of 0.95, strong correlation is observed also with RS of 0.8. Moderate correlation is shown with Comp.

PC3, which primarily explains SPAD variability, is significantly influenced by the two-way interaction between root zone temperature and irrigation timing. In case of cooler temperature, Spad (reversed PC3) was increased in seedlings irrigated at morning. On the opposite, heated seedlings showed increased Spad when irrigated at evening. Figure 5 shows how root zone temperature modulates the effect of irrigation on Spad values (Table 2).

Overall, the mass of the whole plant was influenced only by root zone temperature, with plants grown under heated conditions showing a significant increase of about 17% in total dry mass.

Physiological Traits

Physiological traits were analysed using PCA, resulting in two principal components that together explained 94% of the total variance. PC1 accounted for 62% of the variance and was positively associated with A and E and negatively associated with Ci. PC2 explained an additional 32% of the variance and was strongly negatively associated with gs, and to a lesser extent with Ci. The component loadings are presented in Table 3.

Figure 6 shows how experimental factors influenced physiological traits. Confidence ellipses (95%) drawn around treatment groups indicate substantial overlap across all experimental factors, suggesting no distinct separation among treatments in the PCA space. These visual

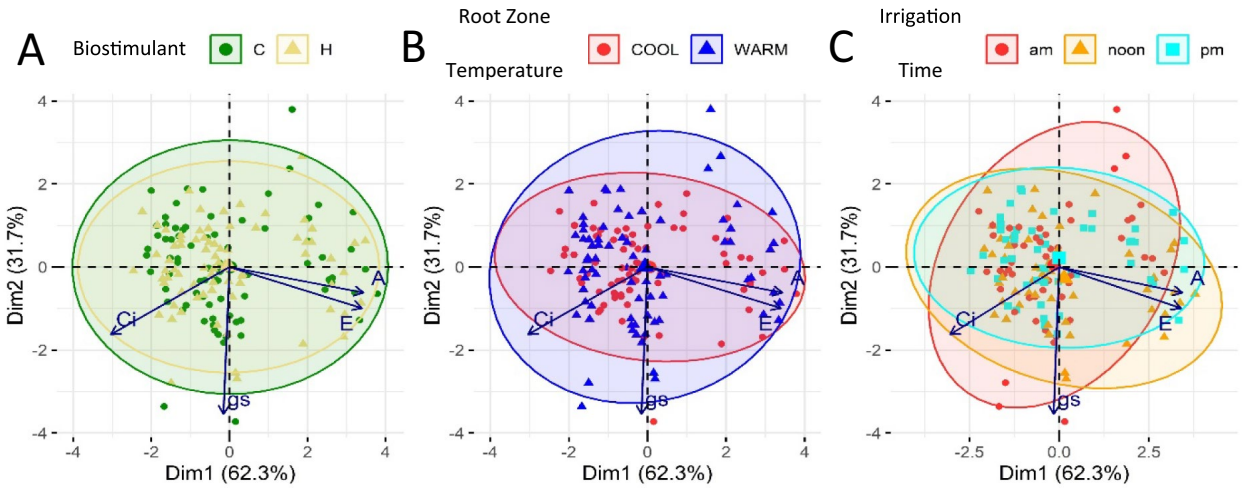


Fig. 6 Principal Component Analysis (PCA) biplot of physiological traits. Each biplot displays PC1 and PC2, which together explain 94% of the total variance. Arrows indicate the loadings of physiological traits. Colours represent groups based on one of three experimental factors, with 95% confidence ellipses drawn around group centroids to illustrate separation in coordinate system. Biplot A shows grouping by levels of biostimulant, B by root zone temperature and C by irrigation timing

Table 4 Physiological parameters of cucumber seedlings grown under two root zone temperature conditions (WARM and COOL), treated with either biostimulant (H) or control (C), across three irrigation timings: morning (am), midday (noon), and evening (pm). Data are presented as mean ± standard deviation ($n = 10$) for the following gas exchange traits: net photosynthesis (A), transpiration (E), stomatal conductance (gs) and intercellular CO_2 concentration (Ci)

T	BS	Irr	A μmol/m^2/s	E mmol/m^2/s	gs mmol/m^2/s	Ci μmol/m^2/s
COOL	**H**	**am**	12.4 ± 1.06	2.51 ± 0.29	213 ± 16.2	463 ± 19.2
		noon	12.2 ± 0.99	2.86 ± 0.36	254 ± 18.8	471 ± 17.8
		pm	11.7 ± 1.21	2.51 ± 0.28	213 ± 10.6	547 ± 22.0
	C	**am**	12.7 ± 0.83	2.64 ± 0.28	259 ± 27.8	474 ± 19.9
		noon	12.4 ± 1.06	2.51 ± 0.29	213 ± 16.2	463 ± 19.2
		pm	12.0 ± 1.16	2.54 ± 0.30	220 ± 16.8	452 ± 23.5
WARM	**H**	**am**	11.3 ± 0.65	2.20 ± 0.18	230 ± 26.8	469 ± 27.0
		noon	13.1 ± 1.37	2.86 ± 0.39	283 ± 26.7	478 ± 23.0
		pm	11.8 ± 0.96	2.42 ± 0.29	212 ± 19.9	462 ± 20.7
	C	**am**	11.2 ± 0.88	2.19 ± 0.17	235 ± 35.0	454 ± 35.9
		noon	12.8 ± 1.26	2.59 ± 0.36	229 ± 22.2	469 ± 19.7
		pm	12.6 ± 1.28	2.60 ± 0.36	227 ± 20.4	466 ± 21.2

Table 5 Principal component loadings of mineral composition

	PC1	PC2
N_{total}	0.1422935	**−0.56332433**
P	**0.3719092**	**−0.34735824**
K	**0.4004105**	−0.28081938
Mg	**0.4058289**	0.27540222
Ca	**0.4006424**	0.20030777
Fe	**0.4098450**	−0.03673292
Na	0.1335647	**0.58260699**
S	**0.4124120**	0.16247657

observations were supported by ANOVA based on linear mixed-effects models, which revealed no statistically significant effects of any of the experimental treatments and their interactions on the physiological principal components (Table 4).

Mineral Composition

The first two principal components together explained 91% of the total variance in the nutrient data, with PC1 accounting for 61% and PC2 for 30%, respectively. The PCA loadings (Table 5) indicated that PC1 was primarily associated with high positive contributions from S, Fe, Mg, Ca, K and P, suggesting this component represents general nutrient accumulation. In contrast, PC2 exhibited strong negative

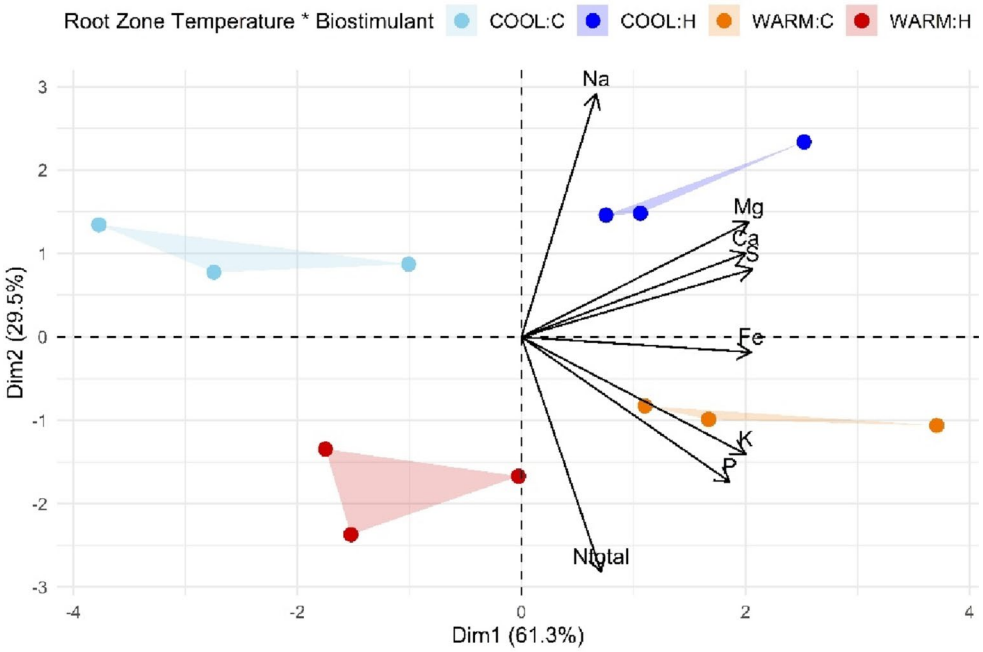


Fig. 7 Principal Component Analysis (PCA) biplot for mineral composition. The biplot displays samples in the space defined by the first two principal components (PC1 and PC2), which together explain 94% of the total variance. Arrows indicate the loadings of individual nutrients on the principal components, with their direction and length reflecting the strength and contribution of each nutrient variable to the PCA axes. Transparent polygons enclose the distribution of samples for each treatment group, warm colours (red and orange) represent warm root zone temperature, while cold colours (cyan and blue) indicate samples grown in cool root zone temperature. Darker colours of these groups (red and blue) represent plants treated with biostimulants and lighter colours (orange and cyan) represent control

Table 6 Nutrient composition and use efficiencies of cucumber seedlings grown under two root zone temperature conditions (WARM and COOL), treated with either biostimulant (H) or control (C), across three irrigation timings: morning (am), midday (noon), and evening (pm). Data are presented for the following traits: total nitrogen (N_{total}), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), iron (Fe), sodium (Na), and sulfur (S), along with water use efficiency (WUE) and nitrogen use efficiency (NUE)

T	BS	lrr	$N_{total\ (\%)}$	*P (%)*	K (%)	Mg (%)	Ca (%)	Fe (%)	Na (%)	S (%)	WUE (gDM/mm)	NUE (gDM/gN)
COOL	**H**	**am**	4,14	1,25	5,61	0,972	2,86	0,015	0,099	0,73	3,85	24,15
		noon	4,34	1,202	5,5	0,962	3,07	0,014	0,101	0,71	3,79	23,04
		pm	4,05	1,267	5,55	1,055	2,93	0,016	0,103	0,83	3,53	24,69
	C	**am**	4,26	1,185	5,18	0,904	2,64	0,013	0,09	0,7	3,56	23,47
		noon	4,09	1,05	4,88	0,84	2,48	0,013	0,081	0,62	3,87	24,45
		pm	3,93	0,959	4,39	0,839	2,23	0,013	0,081	0,62	3,58	25,45
WARM	**H**	**am**	4,92	1,243	5,53	0,826	2,39	0,014	0,064	0,6	3,18	20,33
		noon	4,58	1,351	5,64	0,879	2,55	0,015	0,062	0,68	3,33	21,83
		pm	4,42	1,271	5,36	0,85	2,34	0,013	0,06	0,67	3,58	22,62
	C	**am**	4,76	1,429	5,92	0,954	2,83	0,015	0,082	0,72	2,98	21,01
		noon	4,39	1,389	5,9	0,927	2,74	0,015	0,069	0,74	3,23	22,78
		pm	4,68	1,487	6,59	1,004	3,01	0,016	0,075	0,83	3,53	21,37

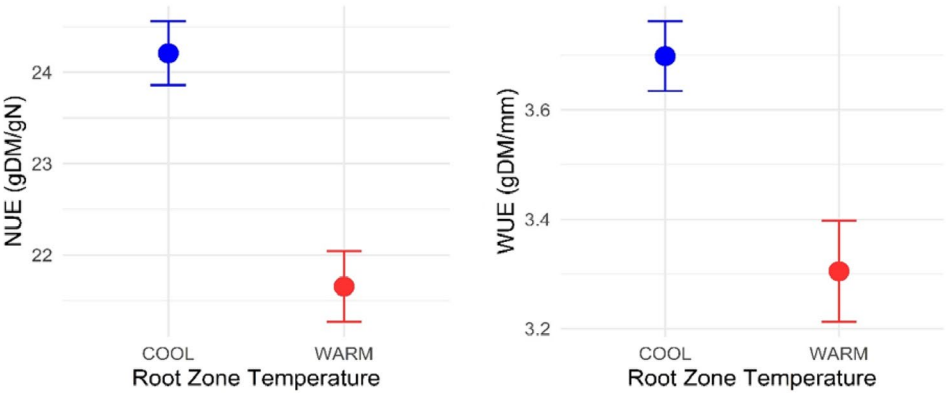


Fig. 8 Mean WUE (right) and NUE (left), ± standard errors across different root zone temperatures. Colours distinguish between root zone temperature (blue for COOL and firebrick red for WARM)

loadings from N_{total} and P and a strong positive loading from Na, indicating a gradient from essential nutrient content to sodium accumulation.

For PC1, the interaction between T and BS was highly significant ($p=0.0007$), while neither main effect was significant. Addition of biostimulant in warm root zone suppressed accumulation of nutrients associated with PC1 in cucumber seedlings, while the opposite can be observed in cool root zone, where biostimulant increased accumulation of these elements in comparison with the control group.

For PC2, the effect of root zone temperature was highly significant ($p<0.001$) as well as its interaction with biostimulant application ($p=0.0083$). Nevertheless, the difference between biostimulant and control is not significantly different in warm or cool root zone when we talk about PC2. Results are graphically displayed in Fig. 7. Nutrient composition per treatment is shown in Table 6.

Use Efficiency

The analysis revealed a significant influence of the main effect of root zone temperature, both for WUE ($p=0.012$) and NUE ($p=0.0008$). In contrast, there was no effect of biostimulant, and their two-way interaction with temperature.

Post hoc comparisons showed that plants grown under cooler temperatures had significantly higher WUE for about 11% and NUE for about 14% compared to those grown in warmer temperature conditions, with a mean difference of 0.393 gDM/mm (from 0.237 to 0.55, $p=0.012$) and 2.55 gDM/gN (from 1.23 to 3.87, $p=0.002$). Means are graphically shown in Fig. 8.

Discussion

This study investigated the potential of a humic acid based biostimulant to enhance the morphological, physiological, and compositional quality of cucumber seedlings under varying growing conditions, specifically root zone temperature and irrigation timing. Overall, root zone temperature emerged as the primary environmental factor affecting plant traits, while irrigation timing had moderate, context-dependent effects. The biostimulant revealed its temperature-dependent mode of action.

For the following discussion it is important to take light conditions into account. Light is strongly correlated with temperature, and together, they regulate plant development (Li et al. 2022). Inside greenhouse, light intensity can be reduced by up to 50% along with chages in light quality, particularly a reduced red to far-red light ratio (Faust et al. 2005). It is evident from Fig. 1 that the daily light integral (DLI) averaged around 12 ± 5 mol m^{-2} day^{-1} in the open area, whereas conditions inside the greenhouse differed significantly. Low light conditions combined with high temperatures promote increased plant height. Although, Cui et al. (2021) concluded that the optimal DLI under artificial lighting conditions was 6.35 mol m^{-2} day^{-1}.

Even soil temperature follows the sun's angle, peaking at midday and declining afterwards. In potted substrates, soil temperature exhibits more dynamic fluctuations than in field conditions, where daily temperature variation diminishes with soil depth (Adams and Zeleke 2017). From Fig. 1, it is clearly evident that cold water lowers soil temperature at the time of irrigation. However, in the case of morning and noon irrigation, solar radiation heats the root zone back up, whereas evening irrigation causes a rapid loss of accumulated heat, and the temperature does not rise again until the next day in unheated treatments. In heated root zone, irrigation causes a sharper drop in temperature, but timing has a less pronounced effect due to artificial temperature control. Temperature modulation via irrigation was previously discussed by Ismail et al. (2007), who reported that proper irrigation timing and frequency could increase tomato yield by 20%.

In our case, irrigation significantly improved root traits at noon irrigation, compared to morning irrigation. This could be attributed to the synchronization of soil moisture availability with peak of plants water demand. This decreases root zone temperature during the hottest part of the day and improves water availability during the driest part of the day, when plants experience their highest hydraulic demand, which peaks around noon (He et al. 2020).

Our findings align with those of Adams and Zeleke (2017) on grapevines, who reported that midday irrigation supports better water infiltration and moisture retention in the root zone, while also influencing temperature. Therefore, it was expected that the effect of irrigation timing would be modulated by root zone temperature, as observed for shoot traits, although the effect was only marginally significant ($p=0.0495$).

It is important to note that the irrigation method used in this study (immersing trays in water until fully soaked to ensure uniform substrate moisture) differs from typical horticultural practices, where overhead irrigation is more common to reach a desired root traits (Franco and Leskovar 2002). Excessive soil water content, however, can inhibit root development (Jang et al. 2020).

Soil temperature is widely recognised as an important ecological determinant of a variety of plant traits (Nievola et al. 2017). The applied root zone temperatures largely remained within the optimal physiological range for cucumber seedlings (around 25 °C), which is generally not associated with severe stress responses (Al-Rawahy et al. 2018; Ding et al. 2016). However, short-term temperature

fluctuations during the warmest and coolest periods of the day may have temporarily approached sub- or supra-optimal thresholds, potentially affecting root metabolic activity and oxygen availability in the rhizosphere (Ding et al. 2016; Zamljen and Slatnar 2024; Tachibana 1987). Such transient deviations could influence plant response and should therefore be considered.

The PCA of morphological data revealed that shoot and root traits were inversely affected by root zone temperature. PC1, which reflected shoot traits, was significantly higher in seedlings grown under warm root zone conditions, indicating enhanced aboveground biomass. Conversely, root biomass, captured by PC2, was reduced in warm conditions compared to unheated root zone, suggesting a trade-off between shoot and root development depending on thermal conditions.

Our results are consistent with (Gosselin and Trudel 1985), where they observed optimal cucumber **shoot growth** at root zone temperatures of 25 °C and 30 °C. However, this doesn't directly explain overall seedling quality. To provide a more comprehensive assessment, the root to shoot ratio, compactness, and Dickson's quality index or other modifications offer a more informative and synergistic evaluation of seedling quality (Binotto et al. 2010; Jang et al. 2020; Niu et al. 2022; Yan et al. 2023).

It was observed that root characteristics are strongly correlated with overall seedling quality indicators, such as root to shoot ratio, compactness and Dickson's quality index. These findings support the conclusion that favourable root traits play a central role in determining morphological seedling quality (Qin and Leskovar 2020). Interestingly, compactness, a shoot-based trait, was more closely associated with root traits (PC2) and only weakly related to shoot traits (PC1). Soil thermal regime has a profound impact on root growth and morphology, as previously noted by Craig et al. (2025), who argued that soil warming may negatively affect root characteristics such as growth, morphology, and longevity.

Morphological observations are closely linked to our mineral and physiological findings. Pregitzer and King (2005) explained that temperature affects water viscosity, soil processes such as decomposition and nutrient mineralisation, plant functioning, and nutrient uptake kinetics. Low temperatures reduce transpiration, thereby decreasing the mass flow of nutrients to the root surface. However, we observed no significant physiological response to any of the experimental treatments. This lack of effect may indicate that uniform light and humidity conditions in the leaf area may have masked subtle physiological responses, that would typically vary under different root zone temperature regimes, as others report (Yamori et al. 2022). However, temperature did not affect Ca concentration. Knowing that Ca transport in plants occurs mainly via the xylem, driven by the transpiration stream due to its phloem immobility (Yu et al. 2024), supports our physiological observations.

We also need to take into account that temperature affects enzyme activity, protein function, and membrane fluidity, all of which influence nutrient acquisition (Bassirirad 2000; Zhang et al. 2006; Kasamo 2003; Palmgren 2001; Lapshin and Trofimova 2025). Bassirirad (2000) commented that these processes can improve nutrient status of plants, despite decreased root traits. These conclusions are consistent with our results. Many studies reported similar results. Craig et al. (2025) found improved assimilation of NO_3, PO_4, and NH_4. Miao et al. (2023) observed higher concentrations of K, N, Ca, and Mg in tomato leaves. Yan et al. (2012) also noted better nutrient absorption at 20 °C compared to 10 °C, despite decreases in root mass and root to shoot ratio.

These results indicate that root zone temperature is a key determinant of both nutrient profiles and root traits. All findings contribute to the proposed explanation that nutrient absorption efficiency and root growth can compensate for each other. Smaller roots can meet the plant's nutrient demands in warm substrate, where the absorption process is relieved due enhanced enzyme activity, root respiration, membrane transporter functioning, and water properties. In contrast, lower temperatures make nutrient absorption more demanding, so roots grow larger to increase the absorbing surface area. Consequently, plants in warm conditions invest in shoot growth to maximize aboveground productivity and plants grown at lower temperatures maintain smaller shoots because they allocate more resources to root growth to cope with challenging nutrient acquisition. Yan et al. (2012) also suggest that low temperature impairs nutrient transport mechanisms, not only uptake from solution, by showing that low root zone temperature significantly restrains nutrient transport from root to shoot.

It is noteworthy that increased nitrogen assimilation under warm root zone conditions was accompanied by a decrease in nitrogen use efficiency (NUE). Similarly, water use efficiency (WUE) declined due to enhanced plant transpiration and increased soil evaporation.

Things started to get interesting when we added humic acid-based biostimulant. Although other studies report that humic substances can induce root growth - speculatively due to their hormone-like activity (Canellas and Olivares 2014), improve SPAD (Pérez-Labrada et al. 2023) and shoot growth (Shalaby & El-Messairy, 2018), we didn't observe similar effects. However, their application did increase aboveground tissue nutrient concentrations, especially under lower temperature conditions.

These results are consistent with the meta-analysis conducted by Rose et al. (2014), which showed that, despite an average growth increase of 15–25% across studies,

many report less than a 5% increase in shot or root biomass response to humic substances, indicating that responses are highly variable and often moderated by environmental factors, as demonstrated in our study.

It is important to understand that, even if humic acids didn't affect plant morphology directly, the beneficial effects on transplants could last longer, even after field establishment, as was observed by Qin and Leskovar (2020). Although our seedlings showed no observable differences in morphology or physiology after biostimulant application, they were more agile in nutrient acquisition, which can benefit during the field establishment or even later, in terms of increased yield and its quality, as demonstrated by Zamljen et al. (2024) on snack pepper.

Based on the multifaceted capabilities of humic acids and previously reported temperature related effects, it can be interpreted that humic acids may exhibit a temperature-dependent window in which their biostimulant efficiency is maximized. Humic acids are known to improve plant nutrient acquisition through mechanisms such as the stimulation of plasma membrane H^+-ATPase activity and other enzymes involved in nutrient acquisition (Mora et al. 2010). Activation of H^+-ATPase increases proton extrusion into the rhizosphere which facilitates nutrient solubilization and promotes ion uptake (Palmgren 2001). By improving nutrient status, these processes can indirectly enhance plant metabolic efficiency and contribute to resilience under environmental constraints (Bassirirad 2000; De Pascale et al. 2017). Consequently, others observed increased Na, K (Akinci et al. 2009), Ca, Fe, S, Mg, P, Zn, K, Cu, Mn in plants treated with humic substances (Denre et al., 2014). As mentioned before this enzymatic activity is temperature-dependent (Ahn et al. 2000), suggesting that plant characteristics should be in tune with biostimulant to achieve desirable effect.

This pattern can be clearly observed in the case of Fe, S, Ca, and Mg concentrations. While temperature alone does not drastically influence their concentration, a notable interaction occurs with the biostimulant, which increases their concentration only under cool root zone conditions. In addition to other elements, for Fe assimilation, cucumbers employ a strategy involving the reduction of Fe^{3+} to the more soluble Fe^{2+}, accompanied by rhizosphere acidification and subsequent transport into root epidermal cells via high-affinity iron transporters (Gao and Dubos 2021). Humic acids can enhance Fe acquisition by modifying root exudation profiles, inducing temporal alterations of involved enzyme activity and gene expression, and by forming soluble Fe-HS complexes that are directly available to plants (Canellas and Olivares 2014; Pérez-Labrada et al. 2023; Zuluaga et al. 2023). While humic acids have been reported to enhance N uptake (Canellas and Olivares 2014), no such effect was observed in our experiment, where N concentration is clearly temperature modulated.

A prior study by Niu et al. (2022) also confirmed the temperature-dependent biostimulant performance, supporting the hypothesis that biostimulants require specific thermal conditions to exert their full biostimulant potential. Thus, the interaction between temperature and humic acids observed in our trial may reflect a functional threshold or optimal temperature range, beyond which their effects are either limited or altered.

Humic acids can partly improve plant performance by enhancing soil conditions. For example, Fe is less available in soils with low organic matter content, alkaline reaction, and high carbonate–bicarbonate concentrations (Pérez-Labrada et al. 2023). Interestingly, they may still provide benefits even in seedling production, where substrates are already carefully selected, plants are regularly fertigated and irrigated to maintain optimal growing conditions. Humic-based products can further improve vegetable seedling quality traits, offering added value in controlled production systems (Qin and Leskovar 2020).

Conclusions

Our findings highlight that, among all factors examined, root zone temperature plays the primary role in cucumber seedling development, affecting morphological traits and nutrient accumulation. Cooler root zone temperatures promoted more balanced root and shoot growth, while warmer conditions favoured shoot biomass at the expense of roots. Although irrigation timing had some effect, it is likely that irrigation time would be more determinant if plants were grown for a longer period. A key contribution of this work is the finding that the effectiveness of humic acid-based biostimulant is temperature-dependent—a relationship previously only hypothesized in the literature. Our findings fill this gap, providing the groundwork for future research on tailoring biostimulant use to specific environmental conditions.

Seedlings that achieved the best quality and exhibited more efficient resource utilisation due to lower root zone temperature showed additional improvement in nutritional content following biostimulant application. Even in carefully managed seedling production systems, where environmental variables are tightly controlled, humic substances may offer meaningful benefits that extend beyond early growth stages, potentially improving field establishment, yield, and crop quality.

Interestingly, even cucumbers, known for their preference for warm conditions, can benefit from avoiding excessive root zone temperatures. These findings underscore the

importance of optimizing both temperature conditions and biostimulant application to fully unlock their potential in early seedling development.

While seedling morphology and physiological traits were largely unaffected, the observed effects on nutrient uptake highlight a potentially important mechanism of biostimulant action. Future studies should expand nutrient profiling to include a broader range of micronutrients in order to further refine our understanding of temperature-dependent biostimulant responses. Significant research gaps remain in elucidating how humic substances interact with other environmental drivers, such as extreme temperatures, drought or salinity and how these interactions influence stress-related metabolites and biochemical pathways. Future research should therefore prioritise integrative experiments that combine comprehensive nutrient analysis with stress and metabolic indicators under precisely controlled yet agronomically realistic conditions, providing a foundation for evaluating humic acid performance under complex, real-life production scenarios involving multiple interacting stress factors.

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Authors' Contributions K.P. Conceptualization, Data curation, Software, Visualization, Writing – original draft, Formal analysis; A.S. Methodology, Resources, Supervision, Conceptualization, Writing – review and editing.

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Data Availability The datasets generated during the current study are available in the Repository of the University of Ljubljana, [https://repozitorij.uni-lj.si/IzpisGradiva.php?id=175467&lang=slv].

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for Publication Not applicable.

Ethics Approval and Consent to Participate Not applicable.

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