



Article

Comparative Effects of GABA, 5-Aminolevulinic Acid, and *Bacillus*-Based Treatments on IBA-Pretreated Tea Chrysanthemum Cuttings Under Plateau Cultivation Conditions

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Abstract

Plateau environments are characterized by low temperatures, intensive radiation and drastic diurnal temperature fluctuations, all of which greatly suppress rooting and seedling establishment of tea chrysanthemum cuttings. To compare the regulatory effects of different rooting regulators on tea chrysanthemum cuttings under plateau cultivation conditions, this study conducted a 60-day plug-tray cultivation experiment from late July to late September with three biological replicates. All the cuttings, including the IBA-pre-treated control (CK), were uniformly pretreated with 500 mg·L⁻¹ indole-3-butyric acid (IBA). On this basis, four treatments, including 5-aminolevulinic acid hydrochloride (5-ALA, T1), γ -aminobutyric acid (GABA, T2), *Bacillus amyloliquefaciens* (T3) and *Bacillus velezensis* (T4), were applied to determine root morphology, seedling growth, physiological stress metabolism, photosynthetic capacity and rhizosphere substrate characteristics. The different rooting promoters exerted distinct regulatory effects on cutting performance. The GABA treatment significantly improved leaf gas exchange, seedling growth, antioxidant status and rhizosphere nutrient conditions, with net photosynthetic rate, stomatal conductance and transpiration rate improved by 316.34%, 92.31% and 168.00%, respectively, and significantly increased seedling vigor index, plant height and stem diameter by 21.74%, 60.85% and 46.08%, respectively. It also elevated the soluble sugar content and the ascorbate peroxidase (APX) activity, reduced malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) accumulation, and optimized rhizosphere available nitrogen and phosphorus levels, as well as related enzyme activities. Nevertheless, the 5-ALA treatment exhibited unique advantages in improving rooting rate and seedling survival. Mantel tests confirmed that the seedling vigor index closely correlated with the root architecture, the total chlorophyll and the transpiration rate. A principal component analysis (PCA) and a cluster heatmap both identified GABA as the treatment with relatively balanced overall performance. A comprehensive D-value evaluation ranked the treatments as GABA > *B. amyloliquefaciens* > 5-ALA > *B. velezensis* > control. The treatment with 5-ALA mainly improved the antioxidant capacity, the *B. amyloliquefaciens* treatment favored root elongation and total nutrients, and the *B. velezensis* treatment only produced mild improvements. This study indicates that the different exogenous regulators target divergent growth and physiological processes, and the GABA treatment could coordinately boost root development, photosynthetic performance, antioxidant defense and rhizosphere nutrient cycling, thus presenting great application potential for tea chrysanthemum cutting propagation under plateau cultivation conditions.



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Keywords: antioxidant enzyme activities; cutting propagation; γ -aminobutyric acid (GABA); photosynthesis; plateau conditions; rhizosphere nutrients; tea chrysanthemum

1. Introduction

Tea chrysanthemum (*Chrysanthemum morifolium*), a perennial herb, is well known for its ornamental, edible and medicinal applications [1]. Its flowers are commonly processed into herbal tea and are rich in bioactive constituents including chlorogenic acid and flavonoids, granting the crop considerable uses [2]. Tea chrysanthemum has become an economically important horticultural crop in China. For instance, Tongxiang City in Zhejiang Province, one of the major tea chrysanthemum-producing areas in China, has a cultivation area of 3161 ha and an annual yield of 9090 metric tons [3]. Driven by an increasing market demand, tea chrysanthemum cultivation has expanded rapidly in recent years, with high-quality seedling production becoming a top industrial priority [4]. The employment of cuttings is currently the prevailing approach for the commercial mass propagation of tea chrysanthemum, owing to its straightforward operation, rapid seedling growth, low cost, and reliable maintenance of elite parental characteristics [5–8]. Root development is a decisive factor for cutting survival, seedling vigor and post-transplant performance. Accordingly, improving rooting competence and root quality is fundamental to achieving efficient tea chrysanthemum production.

Rooting-promoter treatments provide an effective approach to improve root development and cutting quality. Rooting-promoter treatments are agrochemical formulations widely employed in horticultural seedling production to stimulate root initiation, enhance root activity, and improve rhizosphere conditions [9]. Previous work has documented the variable effects of different rooting-promoter types on cutting rooting and seedling growth. Chemical rooting promoters such as 5-aminolevulinic acid (5-ALA) and γ -aminobutyric acid (GABA) improve plant performance by boosting chlorophyll synthesis, photosynthetic capacity [10–12], stress tolerance, osmotic balance, and antioxidant defense [13–16]. Microbial rooting promoters including *Bacillus velezensis* and *Bacillus amyloliquefaciens* enhance seedling vigor via secretion of bioactive metabolites, nutrient mobilization, and rhizosphere optimization [17,18]. Unfortunately, most of the current rooting-promoter treatment studies focus on lowland plain systems. Few systematic comparative studies evaluate diverse rooting-promoter treatments under high-altitude plateau conditions.

Plateau ecological conditions feature low temperatures, relatively intense solar radiation and large diurnal temperature fluctuations [19–21]. Tea chrysanthemum cuttings grown under high plateau conditions frequently exhibit poor rooting, physiological dysregulation and reduced stress tolerance. These factors impair cutting survival and seedling quality, and as a result constitute a major constraint on large-scale tea chrysanthemum propagation at high altitudes. Although diverse rooting-promoting substances have proven effective for chrysanthemum cuttings under low-altitude conventional environments, comparative evaluations of chemical and microbial rooting promoters for tea chrysanthemum under combined plateau conditions remain scarce. Accordingly, the present study was carried out under plateau conditions, aiming to evaluate how four different rooting promoters modulate the rooting performance, the physiological traits, and the substrate properties of tea chrysanthemum cuttings and to screen out promising treatments via a comprehensive multi-indicator assessment. This work intends to provide theoretical and technical support for efficient tea chrysanthemum seedling production in plateau regions. It should be noted that without low-altitude control groups, we cannot fully separate the plateau environment

effects from the general cultivation effects; further comparative experiments are needed in future investigations.

2. Materials and Methods

2.1. Plant Materials and Experimental Site

The tea chrysanthemum (*Chrysanthemum morifolium*) cultivar ‘Nannong Jinju’ was used in this study. The cultivar was provided by Nanjing Shanghui Horticulture Co., Ltd. (Nanjing, China).

These experiments were conducted at the Horticultural Innovation Base of the Academy of Agriculture and Forestry Sciences, Qinghai University. The site is located at 101°45′ E, 36°42′ N, at an elevation of 2320 m above sea level. It has a temperate semi-arid plateau climate, characterized by cold winters, mild summers, and a mean annual air temperature of 5.7 °C. During the experimental period, the greenhouse microclimate was recorded as follows: maximum temperature 32.6 °C, minimum temperature 7.1 °C, and average temperature 17.4 °C.

2.2. Experimental Design

Five treatments were established, each with three biological replicates.

Healthy, vigorous mother plants of tea chrysanthemum ‘Nannong Jinju’ were used for cutting preparation. Uniform semi-lignified shoots at a physiological age of 4–5 weeks were excised from the mother plants, and shoots bearing 3–5 fully expanded leaves were selected as cuttings. Each treatment included three 50-cell plug trays, giving a total of 150 uniform cuttings. The lower leaves were removed, and the cuttings were trimmed to a uniform length with clean basal cuts, leaving approximately three upper leaves. All cuttings across all treatments received identical pre-planting pretreatment: soaking for 5 min in a solution containing carbendazim (50% wettable powder, diluted 1:1000, *w/v*) and indole-3-butyric acid (IBA, 500 mg/L). The IBA-pretreated control (CK) was irrigated with an equal volume of distilled water without additional rooting-promoter agents, while the other groups received corresponding rooting-promoter solutions via irrigation. The cuttings were then inserted vertically into the center of 50-cell plug trays filled with a growth medium consisting of peat moss, perlite, and vermiculite at a ratio of 2:1:1, to a depth of approximately 2 cm. After planting, the trays were thoroughly watered, and any leaning cuttings were gently straightened.

2.3. Test Rooting-Promoter Treatments

Four rooting-promoter treatments were evaluated in this study and classified into two categories: exogenous chemical regulators and microbial formulations.

The exogenous chemical regulators included T1, 5-aminolevulinic acid hydrochloride (5-ALA; final concentration 100 mg/L [22]) and T2, γ -aminobutyric acid (GABA; final concentration 50 mg/L [23]). Both chemicals were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China).

The microbial formulations included T3, a *Bacillus amyloliquefaciens* strain KN-527 formulation (viable cell count $\geq 1 \times 10^{10}$ CFU/mL), diluted 1000-fold with distilled water before application [24], and T4, a *Bacillus velezensis* strain ZF145 formulation (viable cell count $\geq 2 \times 10^8$ CFU/mL), diluted 200-fold with distilled water before application [25]. T3 was purchased from Syngenta (China) Investment Co., Ltd. (Hong Kong, China), and T4 was provided by the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (CAAS) (Beijing, China).

The rooting-promoter treatments were applied twice during the seedling propagation period. The first application was made 7 days after acclimation, and the second application

was 7 days later. For each application, 500 mL of the corresponding working solution was applied to each plug tray by irrigation. The IBA-pretreated control (CK) trays received an equal volume of distilled water at the same time.

2.4. Index Determination

2.4.1. Determination of Root Morphology and Plant Growth Indicators

Root morphological traits were analyzed using a Microtek root analysis system (Hsinchu, Taiwan).

Plant height was quantified as the vertical distance from the stem base to the shoot apex [26]. Stem diameter was measured at the stem base. Rooting rate was calculated as the percentage of cuttings that developed new roots relative to the total number of cuttings. Survival rate was calculated as the percentage of living cuttings relative to the total number of cuttings. The seedling vigor index was calculated as follows: seedling vigor index = (stem diameter/plant height) $\times$ total plant dry weight.

2.4.2. Measurement of Physiological Indicators

Soluble sugar content was detected using the BoxBio reagent kit (Beijing, China). Soluble protein content was quantified via the Coomassie Blue method [27].

Malondialdehyde (MDA) content was assayed according to the protocol established by Heath et al. [28]. Quantification of hydrogen peroxide (H₂O₂) content followed the analytical protocol reported by Patterson et al. [29].

Superoxide dismutase (SOD) activity was quantified via the nitroblue tetrazolium (NBT) assay [30,31]. Peroxidase (POD) activity was assayed via the guaiacol method [32]. Assays for catalase (CAT) activity followed the method in citation [33]. Ascorbate peroxidase (APX) activity was determined using the method described by Nakano et al. (1981) [34].

To determine photosynthetic indicators, fully expanded leaves with ample access to supplementary light were sampled, and the Li-6800 photosynthesis analyzer (Beijing Laikuo Biological Technology Co., Ltd., Beijing, China) was used to record all target photosynthetic parameters.

To measure photosynthetic pigments, chlorophyll content was measured using the 95% ethanol dark extraction method, following the procedures of Tanan et al. [35]. Absorbance was measured at 665 nm, 649 nm, and 470 nm and converted to pigment content using the ethanol system formulas established by Lichtenthaler [36].

2.4.3. Determination of Nutrient Concentrations and Enzyme Activity in the Rhizosphere Substrate

First, rhizosphere substrates distributed around and adhering to tea chrysanthemum roots were collected. Then, pH was measured by the potentiometric method [37]; organic matter was determined by the potassium dichromate oxidation method [38]; total nitrogen was measured by the Kjeldahl method [39]; total phosphorus and available phosphorus were determined by the molybdenum–antimony anti-colorimetric method [40]; total potassium and available potassium were measured by the flame photometry method [41]; available nitrogen was determined by the alkaline hydrolysis diffusion method [42]; and the activities of urease, phosphatase, sucrase, and catalase in the rhizosphere substrates were measured using MuMi assay kits (Guiyang, China).

2.5. Statistical Analysis

WPS Office (version 12.1.0) Excel handled the sorting and arrangement of raw experimental datasets. SPSS 27.0 carried out one-way analysis of variance (ANOVA) paired with Duncan's method, while SIMCA 14.1 delivered principal component analysis (PCA)

calculations. Cluster heatmap analysis was plotted using Origin 2021 software. Mantel correlation heatmaps were generated using an online platform (<https://cloud.metware.cn/>); specifically, the Bray–Curtis distance was used as the distance metric and the number of permutations was set to 999. Schematic diagrams were created using an online platform (<https://www.biorender.com>).

3. Results

3.1. Growth and Root Morphology of Tea Chrysanthemum Cuttings Under Different Treatments

The rooting-promoter treatments significantly modulated the aboveground growth and the root architectural characteristics of the tea chrysanthemum cuttings. Relative to the untreated control group, the cuttings subjected to exogenous rooting promoters displayed enhanced shoot growth and superior root system development. Notably, the T2 and T3 treatments yielded the most pronounced improvements in root traits, with the cuttings producing a more extensive root distribution and denser fibrous root networks (Figure 1).

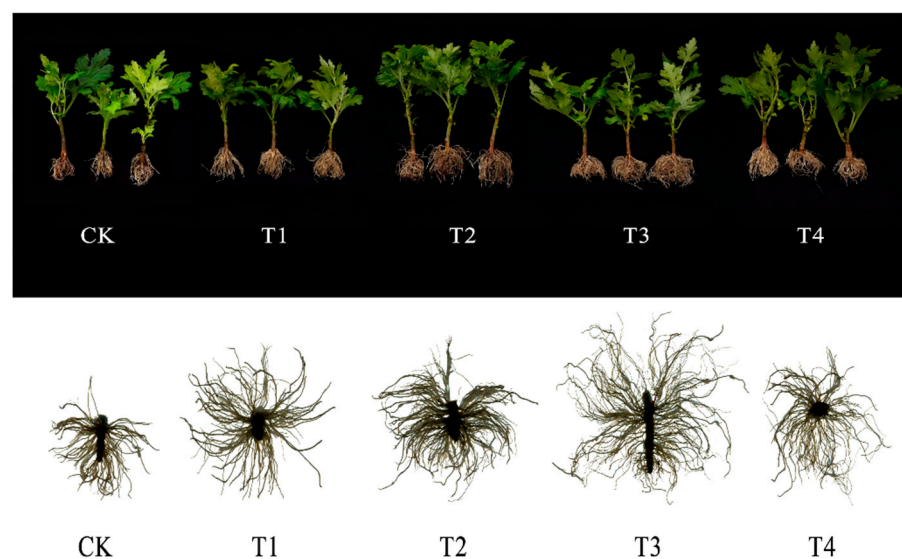


Figure 1. Effects of different treatments on the aboveground morphology and root development of chrysanthemum cuttings. Note: CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ -aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

3.2. Effects of Different Treatments on Root Morphology Indicators

The different rooting promoters significantly altered the root morphological traits of the cuttings (Table 1). Most of the treatments effectively increased total root length, projected area, volume, surface area, tip number, and root activity compared with the control. Quantitative differences existed among the treatment groups. T3 exerted the strongest effects on root elongation and branching, as well as increased total root length, average diameter, and root tip number by 134.48%, 47.33%, and 154.63%, respectively. T1 enhanced root expansion and physiological activity, along with 126.98%, 105.78%, 127.30%, and 160.23% increases in projected root area, root volume, surface area, and root activity. T2 improved root morphology comprehensively, and it increased average root diameter, projected area, volume, tip number, and root activity by 29.63%, 113.65%, 58.99%, 92.68%, and 140.00%, respectively. These results confirmed distinct root response patterns induced by the different treatments. T3 primarily promoted root elongation and branching and T1 improved root expansion and activity, whereas T2 produced a balanced enhancement of multiple root morphological indices.

Table 1. Effects of different treatments on the root morphology indicators of tea chrysanthemum cuttings.

Treatment	Total Root Length (cm)	Average Root Diameter (mm)	Root Projected Area (cm ²)	Root Volume (cm ³)	Root Surface Area (cm ²)	Number of Root Tips	Root Activity (μg·g ⁻¹ ·h ⁻¹)
CK	542.52 ± 17.67 e	2.43 ± 0.20 c	159.43 ± 5.78 d	86.19 ± 1.06 d	500.86 ± 3.91 e	205.00 ± 4.58 e	4.35 ± 0.31 d
T1	1035.38 ± 15.53 b	3.29 ± 0.18 ab	361.87 ± 11.02 a	177.36 ± 9.18 a	1138.47 ± 34.50 a	370.00 ± 11.00 c	11.32 ± 0.35 a
T2	967.26 ± 46.35 c	3.15 ± 0.07 b	340.62 ± 6.13 ab	137.03 ± 2.03 b	928.81 ± 74.12 c	395.00 ± 4.36 b	10.44 ± 0.47 b
T3	1272.09 ± 14.34 a	3.58 ± 0.16 a	335.76 ± 12.95 b	170.31 ± 9.95 a	1054.83 ± 53.78 b	522.00 ± 2.65 a	7.66 ± 0.24 c
T4	663.21 ± 12.30 d	2.74 ± 0.33 c	265.57 ± 21.71 c	122.21 ± 5.06 c	707.54 ± 38.46 d	251.00 ± 16.09 d	7.33 ± 0.33 c

Note: Data are presented as mean ± SD (*n* = 3 biological replicates). Different lowercase letters within each column denote significant differences among treatments based on Duncan's multiple-range test (*p* < 0.05). CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ-aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

3.3. Effects of Different Treatments on Growth Status

The rooting-promoter treatments significantly modified the growth performance of the tea chrysanthemum cuttings, including rooting rate, survival rate, seedling vigor index, plant height, and stem diameter (Table 2). All the applied treatments improved these growth parameters relative to the control group. T1 yielded the maximum rooting rate and survival rate, which were 31.43% and 23.42% higher than those of the control, respectively. In terms of vegetative growth, T2 yielded the greatest plant height, with an increase of 60.85% compared with the control. Both T1 and T2 exhibited similarly improved stem diameters, which increased by 46.99% and 46.08%, respectively, relative to the control. For the seedling vigor index, T2 and T3 showed a comparable and superior performance, with no significant difference between the two treatments.

Table 2. Effects of different treatments on the growth status of tea chrysanthemum cuttings.

Treatment	Rooting Rate (%)	Survival Rate (%)	Seedling Vigor Index	Plant Height (cm)	Stem Diameter (mm)
CK	70.00 ± 3.46 d	74.00 ± 2.00 d	0.23 ± 0.01 c	10.55 ± 0.05 d	3.32 ± 0.03 d
T1	92.00 ± 2.00 a	91.33 ± 3.06 a	0.27 ± 0.01 ab	14.77 ± 0.31 b	4.88 ± 0.02 a
T2	80.67 ± 3.06 b	82.67 ± 1.15 b	0.28 ± 0.01 a	16.97 ± 0.78 a	4.85 ± 0.04 a
T3	80.67 ± 1.15 b	81.33 ± 2.31 bc	0.28 ± 0.01 a	15.37 ± 0.32 b	4.45 ± 0.03 b
T4	75.33 ± 1.15 c	78.00 ± 4.00 cd	0.25 ± 0.02 bc	12.55 ± 0.05 c	4.05 ± 0.04 c

Note: Data are presented as mean ± SD (*n* = 3 biological replicates). Different lowercase letters within each column denote significant differences among treatments based on Duncan's multiple-range test (*p* < 0.05). CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ-aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

3.4. Effects of Different Treatments on the Content of Soluble Substances in the Roots of Tea Chrysanthemum Cuttings

The rooting treatments markedly altered the root soluble sugar and soluble protein concentrations (Figure 2). T2 generated the maximum soluble sugar accumulation, with a 29.41% elevation relative to the control. The soluble sugar levels rose by 9.50%, 6.09% and 2.74% under the T1, T3 and T4 treatments, respectively. For soluble protein, T3 recorded the highest concentration, representing a 260.29% increase over the control; T1 and T2 followed, with respective increments of 119.44% and 113.15%. These findings confirmed T2's superiority in stimulating soluble sugar buildup, while T3 exerted a stronger stimulatory effect on soluble protein accumulation.

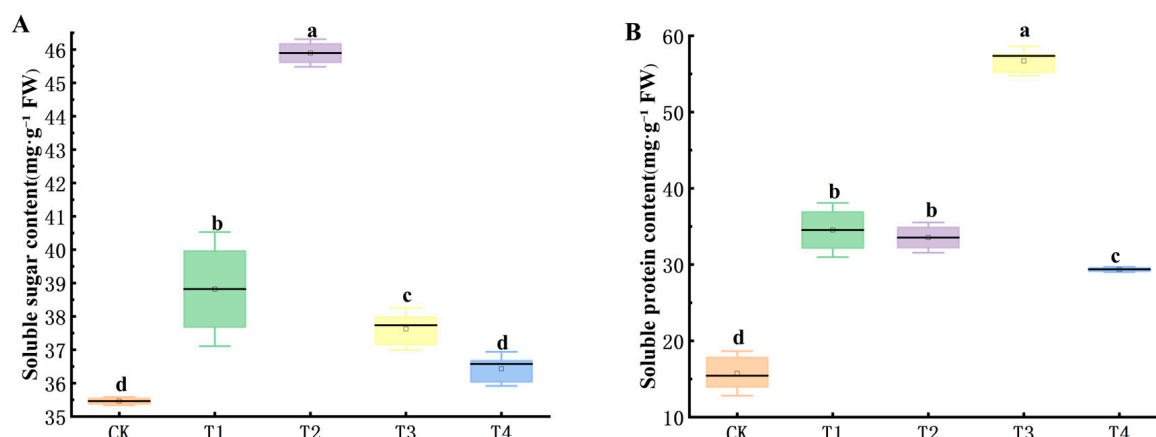


Figure 2. Effects of different treatments on soluble substance contents in roots of tea chrysanthemum cuttings: (A) soluble sugar content; (B) soluble protein content. Note: Values represent mean $\pm$ SD ($n = 3$ biological replicates). Different lowercase letters above bars indicate significant differences among treatments ($p < 0.05$, Duncan's multiple-range test). CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ -aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

3.5. Effects of Different Treatments on the Content of Peroxidized Substances in the Roots of Tea Chrysanthemum Cuttings

All the rooting treatments markedly lowered the root malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations in the tea chrysanthemum cuttings (Figure 3). T2 yielded the minimum MDA and H_2O_2 contents, which declined by 44.34% and 89.72%, respectively, relative to the control. These values were also significantly lower than those of the remaining treatments. T4 exhibited the weakest alleviating effect, with MDA and H_2O_2 reduced by 36.91% and 48.61%, respectively.

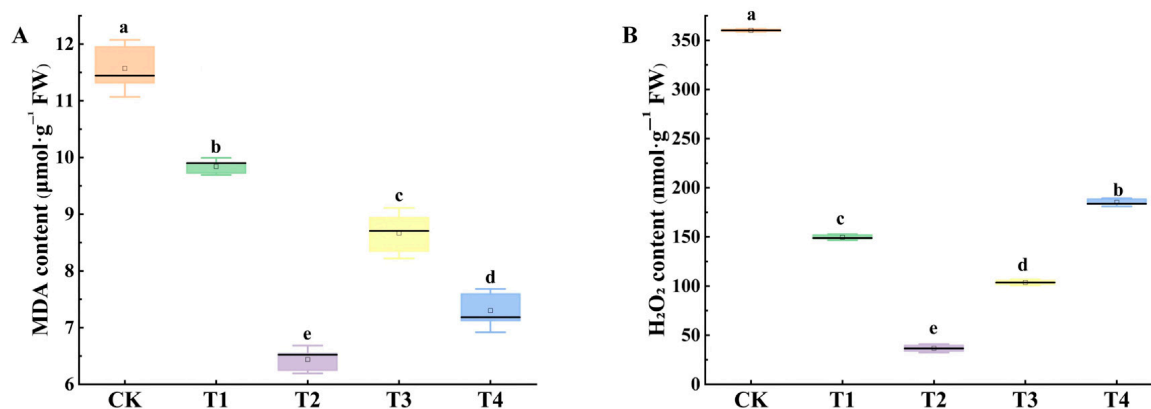


Figure 3. Effects of different treatments on MDA and H_2O_2 contents in roots of tea chrysanthemum cuttings: (A) MDA content; (B) H_2O_2 content. Note: Values represent mean $\pm$ SD ($n = 3$ biological replicates). Different lowercase letters above bars indicate significant differences among treatments ($p < 0.05$, Duncan's multiple-range test). CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ -aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

3.6. Effects of Different Treatments on the Activity of Antioxidant Enzymes in the Roots of Tea Chrysanthemum Cuttings

The rooting treatments substantially modified root antioxidant enzyme activities (Figure 4). T3 and T1 generated the maximum POD and CAT activities, with the POD

activity elevated by 64.04% and 59.50% and the CAT activity elevated by 79.91% and 89.25% relative to the control. T4 exerted milder promotional effects on POD and CAT, with increased POD and CAT increments of 29.83% and 57.32%, respectively. T2 yielded the highest APX activity, which rose by 37.05% compared with the control. The SOD activity was increased following T1 and T3 treatments by 89.74% and 80.57%, respectively. Collectively, T1 and T3 more effectively improved the SOD, POD and CAT activities, while T2 exerted the dominant stimulatory effect on the APX activity.

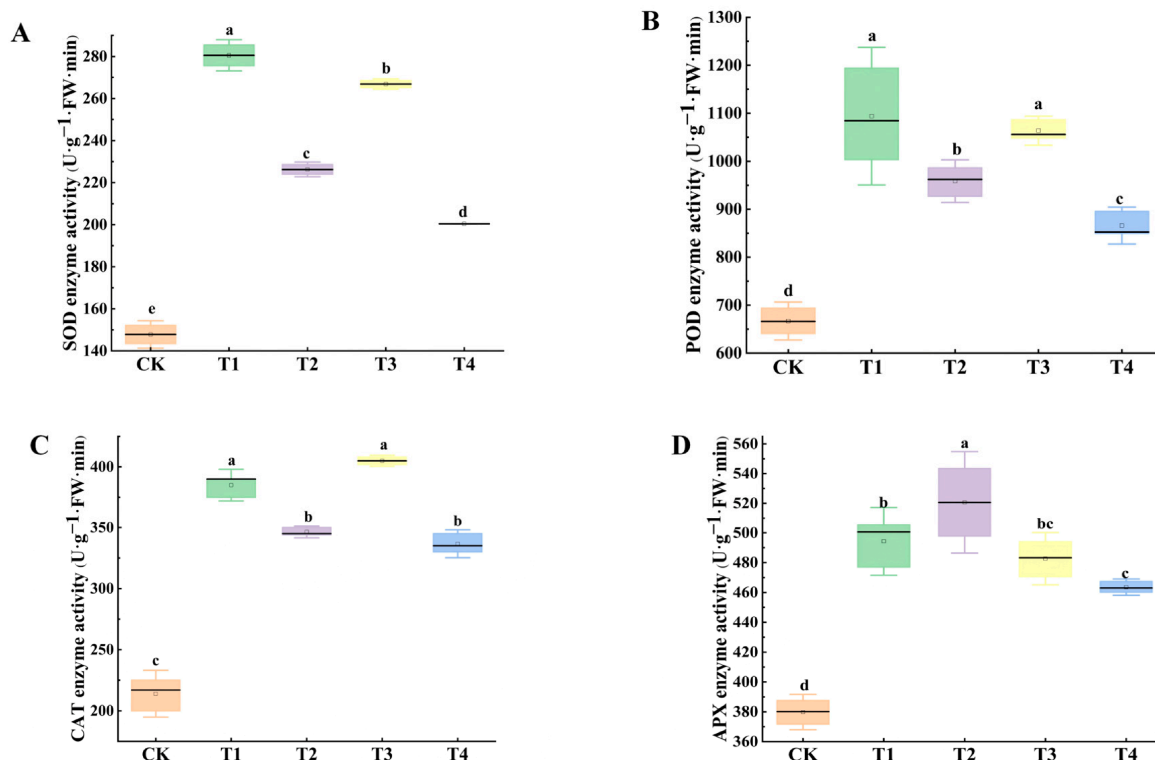


Figure 4. Effects of different treatments on antioxidant enzyme activities in roots of tea chrysanthemum cuttings: (A) SOD enzyme activity; (B) POD enzyme activity; (C) CAT enzyme activity; (D) APX enzyme activity. Note: Values represent mean $\pm$ SD ($n = 3$ biological replicates). Different lowercase letters above bars indicate significant differences among treatments ($p < 0.05$, Duncan's multiple-range test). CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ -aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

3.7. Effects of Different Treatments on the Photosynthetic Pigment Content of Tea Chrysanthemum Cuttings

The rooting treatments markedly altered the leaf photosynthetic pigment levels (Figure 5). All the treatments elevated the chlorophyll content relative to the control. Treatment T3 produced the maximum total chlorophyll, chlorophyll b and carotenoid contents, which rose by 131.82%, 250.00% and 166.67%, respectively. Treatment T4 exerted smaller promotional effects, with total chlorophyll and chlorophyll b increased by 40.91% and 50.00%, respectively. Treatments T1 and T3 produced the highest chlorophyll a concentrations with respective increments of 110.53% and 94.74% over the control, and these values greatly exceeded those of the remaining treatments.

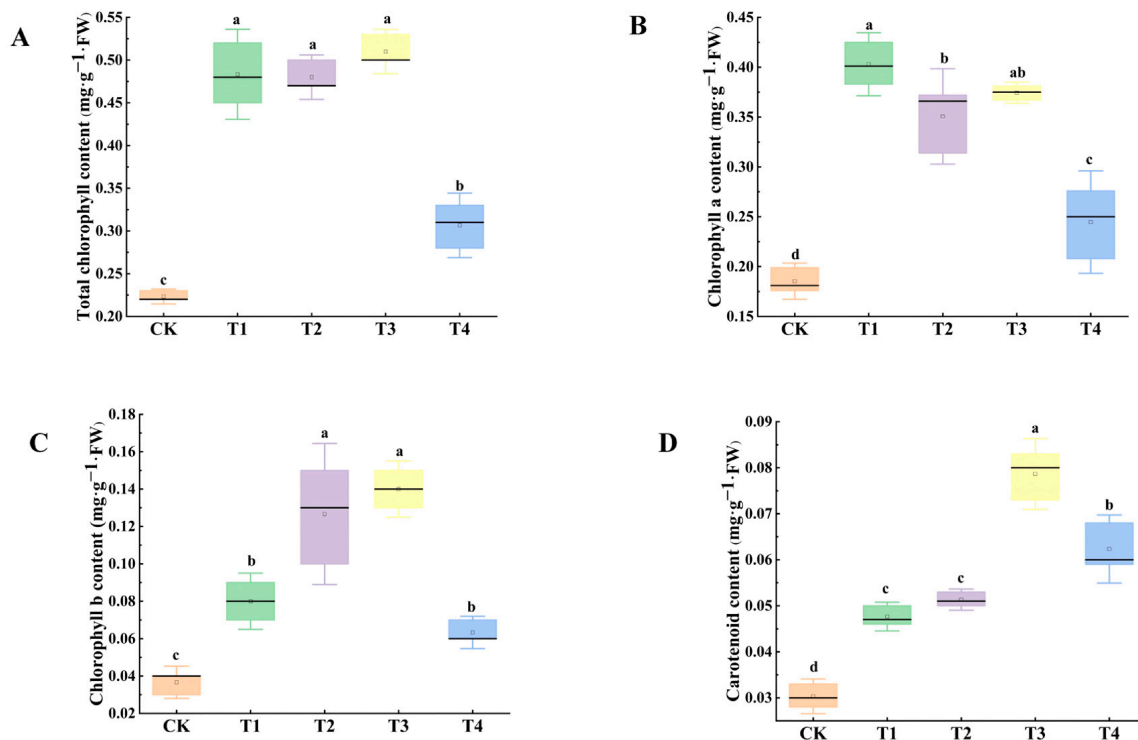


Figure 5. Effects of different treatments on photosynthetic pigment contents in tea chrysanthemum cuttings: (A) Total chlorophyll content; (B) Chlorophyll a content; (C) Chlorophyll b content; (D) Carotenoid content. Note: Values represent mean $\pm$ SD ($n = 3$ biological replicates). Different lowercase letters above bars indicate significant differences among treatments ($p < 0.05$, Duncan's multiple-range test). Note: CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ -aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

3.8. Effects of Different Treatments on Gas Exchange Parameters of Tea Chrysanthemum Cuttings

The rooting treatments substantially modified the leaf gas exchange traits (Figure 6). All the treatments elevated the photosynthetic parameters compared to the control. Treatment T2 showed a higher net photosynthetic rate, stomatal conductance, and transpiration rate than the control, with increases of 316.34%, 92.31%, and 168.00%, respectively. Treatment T1 exerted the second strongest promotional effect on these three indicators, with increases of 190.59%, 76.92% and 160.00%, respectively. Regarding the intercellular CO₂ concentration, treatments T1 and T3 exhibited the largest increases compared to the control, rising by 10.83% and 10.67%, respectively. Treatment T2 was the most effective in increasing the photosynthetic capacity, while treatments T1 and T3 yielded greater improvements in the intercellular CO₂ concentration.

3.9. Effects of Different Treatments on the Physicochemical Properties of Rhizosphere Substrate of Tea Chrysanthemum Cuttings

The rooting treatments markedly reshaped the rhizosphere substrate physicochemical characteristics (Table 3). All the treatments lowered the substrate pH and raised the organic matter, total nutrients and available nutrient concentrations relative to the control, with treatment-dependent response magnitudes. Treatment T4 yielded the minimum substrate pH, followed sequentially by treatments T2 and T3. Treatment T3 resulted in the greatest organic matter, total nitrogen and total phosphorus contents, rising by 14.65%, 8.76% and 8.56%, respectively compared to the control. Treatment T4 generated the highest total potassium and available potassium, with levels 12.43% and 12.31% above the control. Treatment

T2 achieved the maximum level of available nitrogen and available phosphorus, which exceeded the control values by 4.84% and 11.15%, respectively. Collectively, treatment T3 outperformed the other treatments in elevating the organic matter and the total macronutrients; treatment T2 primarily boosted the available nitrogen and phosphorus; treatment T4 exerted the strongest effects on pH decline and potassium accumulation.

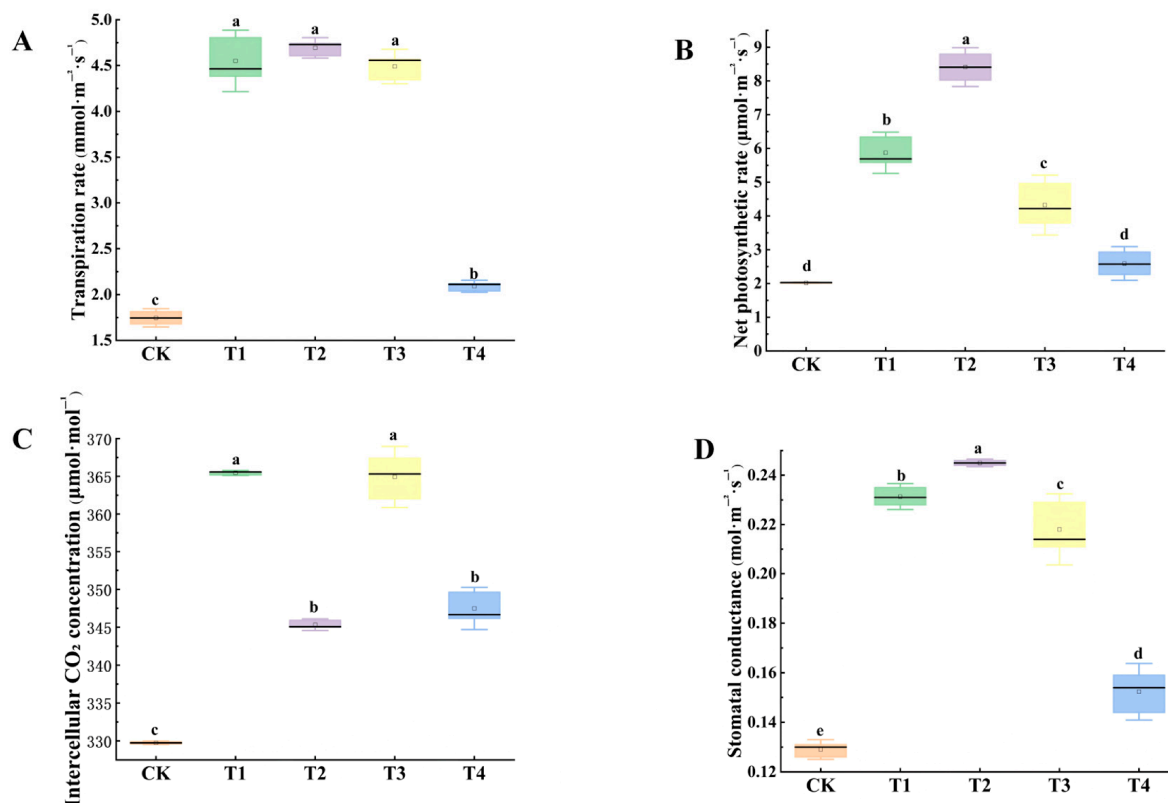


Figure 6. Effects of different treatments on photosynthetic parameters of tea chrysanthemum cuttings: (A) Transpiration rate; (B) Net photosynthetic rate; (C) Intercellular CO₂ concentration; (D) Stomatal conductance. Note: Values represent mean $\pm$ SD ($n = 3$ biological replicates). Different lowercase letters above bars indicate significant differences among treatments ($p < 0.05$, Duncan's multiple-range test). CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ -aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

Table 3. Effects of different rooting-promoter treatments on physicochemical properties of the rhizosphere substrate for tea chrysanthemum cuttings.

Treatment	pH	Organic Matter	Total Nitrogen (%)	Total Phosphorus (g/kg)	Total Potassium (g/kg)	Available Nitrogen (mg/kg)	Available Phosphorus (mg/kg)	Available Potassium (mg/kg)
CK	7.90 $\pm$ 0.20 a	271.02 $\pm$ 11.60 c	1.37 $\pm$ 0.02 d	3.74 $\pm$ 0.05 c	14.24 $\pm$ 0.10 c	998.97 $\pm$ 3.11 c	451.11 $\pm$ 5.09 c	697.95 $\pm$ 3.16 d
T1	7.89 $\pm$ 0.01 a	293.70 $\pm$ 0.59 b	1.47 $\pm$ 0.02 ab	3.90 $\pm$ 0.01 b	15.42 $\pm$ 0.43 b	1000.31 $\pm$ 1.27 c	463.77 $\pm$ 4.85 bc	712.15 $\pm$ 1.53 b
T2	7.84 $\pm$ 0.01 b	272.37 $\pm$ 13.10 c	1.45 $\pm$ 0.02 b	4.05 $\pm$ 0.14 a	15.66 $\pm$ 0.12 ab	1047.30 $\pm$ 20.40 a	501.42 $\pm$ 10.49 a	706.87 $\pm$ 1.09 c
T3	7.85 $\pm$ 0.01 b	310.73 $\pm$ 0.90 a	1.49 $\pm$ 0.01 a	4.06 $\pm$ 0.06 a	15.78 $\pm$ 0.19 ab	1017.74 $\pm$ 1.82 b	471.27 $\pm$ 7.18 b	713.52 $\pm$ 1.61 b
T4	7.82 $\pm$ 0.01 c	297.96 $\pm$ 0.78 ab	1.42 $\pm$ 0.02 c	3.63 $\pm$ 0.02 c	16.01 $\pm$ 0.12 a	1013.99 $\pm$ 1.56 bc	465.33 $\pm$ 8.07 b	783.85 $\pm$ 3.38 a

Note: Data are presented as mean $\pm$ SD ($n = 3$ biological replicates). Different lowercase letters within each column denote significant differences among treatments based on Duncan's multiple-range test ($p < 0.05$). CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ -aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

3.10. Effects of Different Treatments on Rhizosphere Substrate Enzyme Activities of Tea Chrysanthemum Cuttings

The rooting-promoter applications significantly modified the rhizosphere substrate urease, sucrase, phosphatase and catalase activities (Figure 7). All the treatments elevated the four enzyme activities relative to the control to varying extents. Treatment T2 generated the maximum urease and catalase activities, which surpassed the control levels by 13.24% and 3.22%, respectively. Treatment T4 yielded the largest increments in sucrase and phosphatase, increasing by 197.92% and 11.31%, respectively, while treatment T2 increased these two enzymes by 167.11% and 8.37%, respectively.

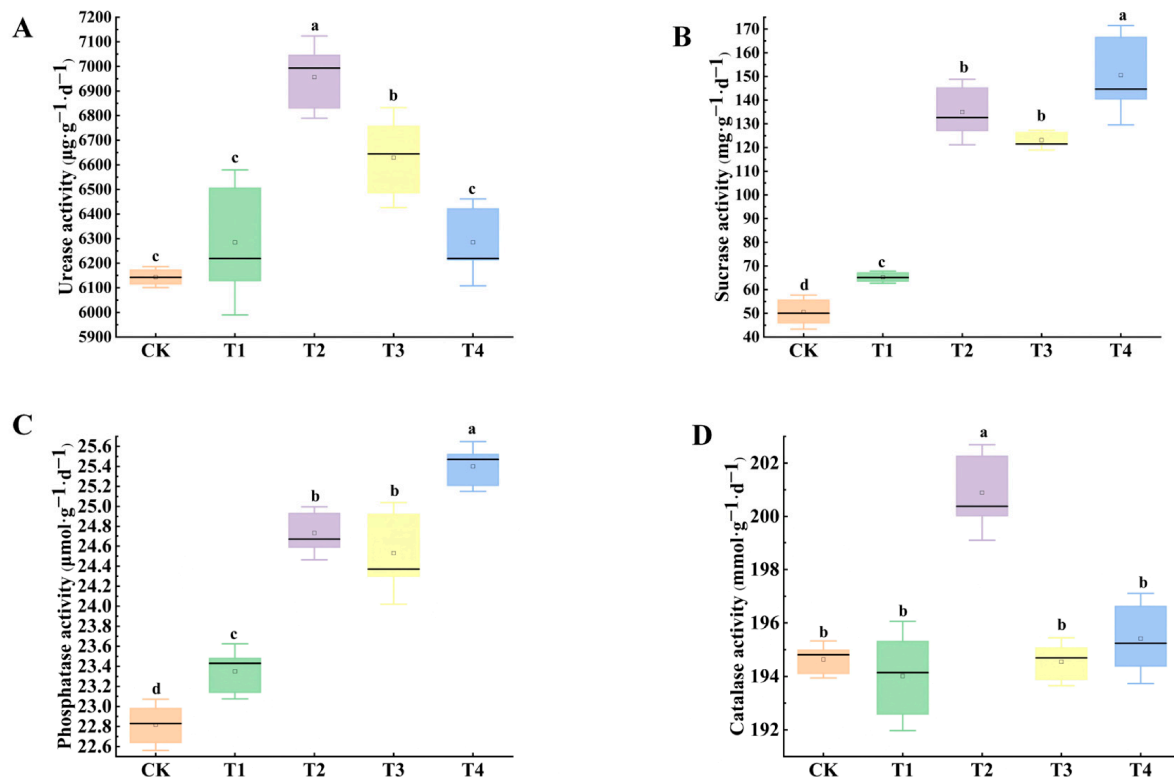


Figure 7. Effects of different treatments on substrate enzyme activity of tea chrysanthemum cuttings: (A) Urease activity; (B) Sucrase activity; (C) Phosphatase activity; (D) Catalase activity. Note: Values represent mean $\pm$ SD ($n = 3$ biological replicates). Different lowercase letters above bars indicate significant differences among treatments ($p < 0.05$, Duncan's multiple-range test). CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ -aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

3.11. Correlation Analysis

The seedling vigor index (SVI) was used as an indicator of the overall growth performance of the tea chrysanthemum cuttings. To identify the traits associated with the SVI, a Mantel analysis was performed to examine the relationships between the SVI and root morphology, physiological traits, and rhizosphere substrate properties.

The analysis revealed strong correlations between the SVI and multiple root and physiological indicators. The total chlorophyll (total Chl), the average root diameter (ARD), the root projected area (RPA) and the transpiration rate (Tr) exhibited the highest correlations with the SVI. These findings confirmed tight connections between seedling vigor, root architecture and photosynthetic pigment buildup, as well as transpiration-associated gas exchange in the tea chrysanthemum cuttings (Figure 8).



Figure 8. Correlation analysis of seedling vigor index with growth physiology and substrate physicochemical properties in tea chrysanthemum cuttings. Note: SVI—seedling vigor index; Chl a—chlorophyll a content; Chl b—chlorophyll b content; Total Chl—total chlorophyll content; Car—carotenoid content; RA—root activity; TRL—total root length; ARD—average root diameter; RPA—root projection area; RV—root volume; RSA—root surface area; RTN—root tip number; PH—plant height; SD—stem diameter; RR—rooting rate; SR—survival rate; SS—soluble sugar content; SP—soluble protein content; Tr—transpiration rate; Pn—net photosynthetic rate; Ci—intercellular CO₂ concentration; Gs—stomatal conductance; OM—organic matter content; TN—total nitrogen content; TP—total phosphorus content; TK—total potassium content; AN—available nitrogen content; AP—available phosphorus content; AK—available potassium content; UE—urease activity; SC—sucrase activity; PHO—phosphatase activity; CATL—catalase activity. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. In the Mantel network, line thickness represents the strength of the correlation, with thicker lines indicating stronger associations between SVI and the corresponding trait groups.

3.12. Principal Component Analysis

A PCA was performed on the growth, physiological and rhizosphere substrate indices to differentiate the five treatments using SIMCA 14.1. The first two principal components explained 77.0% of the total variance (PCA1: 61.5%; PCA2: 15.5%). The score plot clearly separated all the treatments, and the replicates within each group clustered tightly, confirming reliable experimental data. The score and loading plots displayed consistent patterns. The root morphological parameters, the photosynthetic pigments and the SVI clustered near treatments T2 and T3. MDA and H₂O₂ correlated strongly with the control and were distant from the other treatments. Treatment T4 linked to the available potassium (AK), while treatment T1 grouped with the root morphology and antioxidant enzyme indicators (Figure 9).

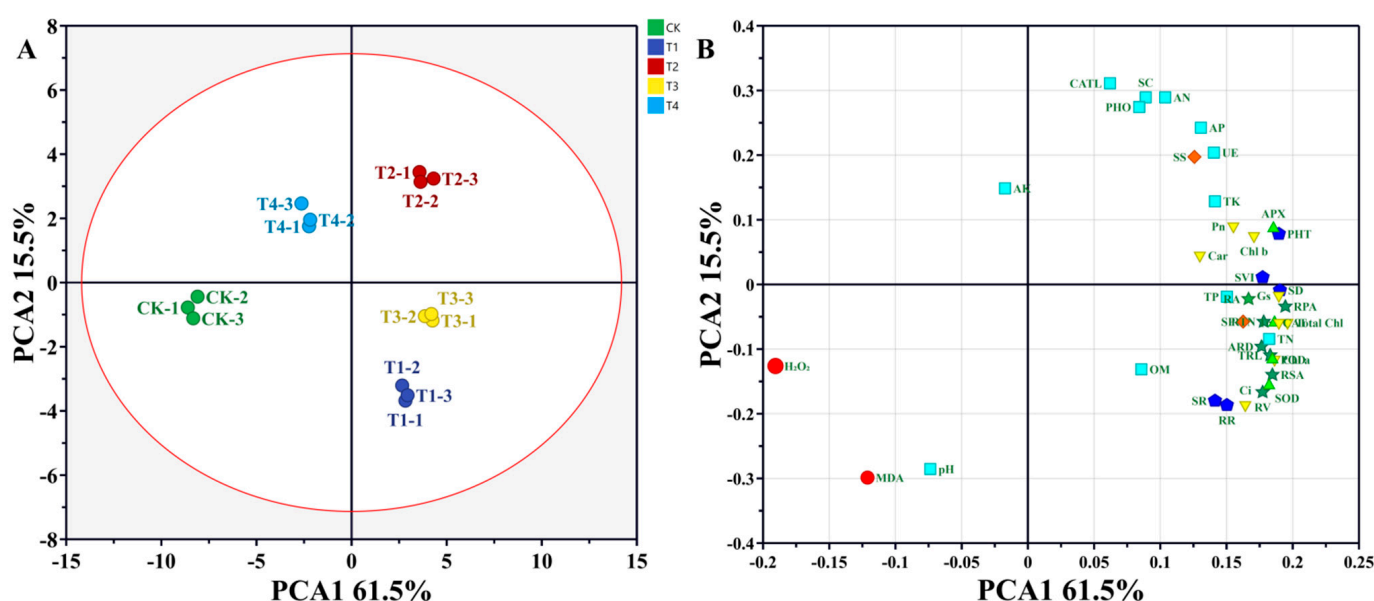


Figure 9. Effects of different treatments on substrate traits and plant growth characteristics: (A) PCA score plot of different treatments; (B) PCA loading plot of the measured traits. Note: PHT—Plant height. CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ -aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

3.13. Comprehensive Evaluation of the Effects of Different Treatments on Tea Chrysanthemum Cuttings

A PCA coupled with weighted normalization was adopted to calculate the integrated D-values for a comprehensive assessment of the growth, physiological and rhizosphere substrate performance across the treatments.

All the treatments achieved superior integrated performance relative to the control. The D-value ranking followed the order T2 > T3 > T1 > T4 > CK (Table 4). Treatment T2 recorded the maximum D-value, 7.55 times higher than the control. Treatments T3 and T1 ranked second and third, with D-values 7.27-fold and 6.09-fold greater than the control, respectively. The control yielded the lowest comprehensive score, which corresponded to inferior cutting growth, physiological status and rhizosphere substrate conditions.

Table 4. Comprehensive evaluation of different treatments on growth physiology and substrate physicochemical properties of tea chrysanthemum cuttings.

Treatment	PC1	PC2	PC3	PC4	μ1	μ2	μ3	μ4	D-Value
CK	−1.56	−0.23	−0.64	−0.55	0.00	0.41	0.16	0.34	0.11
T1	0.51	−1.29	−0.42	1.05	0.89	0.00	0.25	1.00	0.67
T2	0.72	1.28	−1.02	0.05	0.98	1.00	0.00	0.59	0.83
T3	0.77	−0.43	0.71	−1.38	1.00	0.34	0.73	0.00	0.80
T4	−0.44	0.67	1.37	0.83	0.48	0.76	1.00	0.91	0.62

Note: CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ-aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

3.14. Cluster Heatmap Analysis

The cluster heatmap analysis uncovered treatment differences in cutting growth, physiology and rhizosphere substrate traits (Figure 10).

The five treatments split into two clusters: the control and treatment T4 grouped together, while treatments T1, T2 and T3 formed a separate cluster; treatments T2 and T3 clustered closely with similar trait profiles and were clearly distinguished from the control.

The heatmap color gradients revealed obvious treatment discrepancies. Treatment T2 presented higher values for growth indices, photosynthetic parameters, soluble sugar, APX activity and multiple rhizosphere nutrient/enzyme indicators. These results confirmed that treatment T2 comprehensively improved seedling growth, photosynthesis, stress physiology and rhizosphere conditions. By comparison, the control and treatment T4 showed lower overall trait values and weaker performance (we refer the reader to the abbreviation definitions in the legend for Figure 10). The detailed changes in growth, physiological, and rhizosphere substrate traits under treatment T2 are further illustrated in Figure 11.

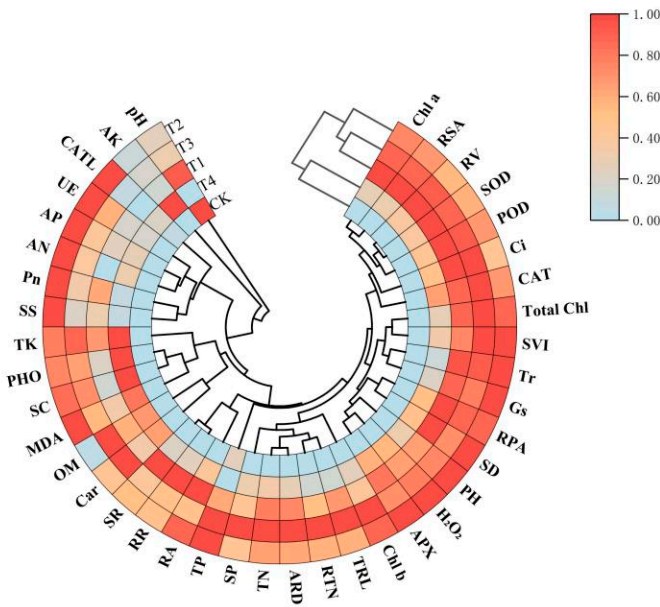


Figure 10. Cluster heatmap analysis of growth physiology and substrate physicochemical properties in tea chrysanthemum cuttings under different treatments. Abbreviations are defined in Figure 8. Note: CK, IBA-pretreated control (water irrigation); T1, IBA-pretreated + 5-aminolevulinic acid (5-ALA) irrigation; T2, IBA-pretreated + γ-aminobutyric acid (GABA) irrigation; T3, IBA-pretreated + *Bacillus amyloliquefaciens* irrigation; T4, IBA-pretreated + *Bacillus velezensis* irrigation.

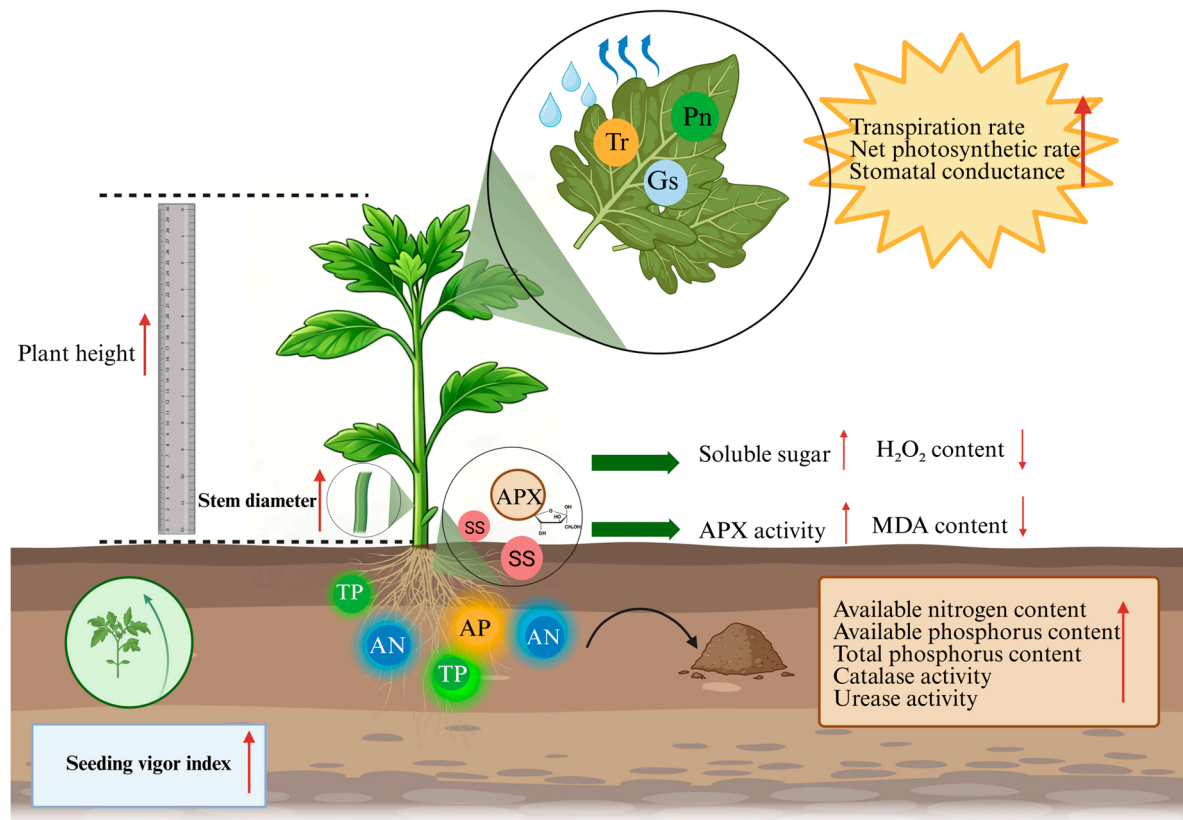


Figure 11. Changes of growth physiology and substrate physicochemical properties of tea chrysanthemum cuttings under treatment T2. Upward and downward arrows indicate increases and decreases in the corresponding parameters, respectively.

4. Discussion

Plateau environments expose plants to intense solar radiation and large diurnal temperature fluctuations, conditions that can constrain plant physiological performance [43]. Low temperatures can further limit adventitious rooting in chrysanthemum cuttings [8]. The successful rooting and high-quality seedling formation of tea chrysanthemum cuttings rely on the coordination of root development, shoot growth, stress tolerance and rhizosphere nutrient supply. In the present study, the four rooting-promoter treatments produced distinct response profiles across these functional levels, indicating that they did not differ merely in the magnitude of a common growth-promoting effect. This divergence is consistent with the fundamentally different nature of the two promoter groups. 5-Aminolevulinic acid (5-ALA) and γ -aminobutyric acid (GABA) are endogenous plant metabolites that, when applied exogenously, can influence plant metabolic and signaling processes [44–48]. In contrast, *Bacillus amyloliquefaciens* and *Bacillus velezensis* are living inoculants whose effects are mediated through interactions with the roots and the rhizosphere [49,50]. Accordingly, the present results are better interpreted in terms of distinct regulatory strategies within the substrate–root–shoot system than as simple differences in the strength of a single rooting response.

For cutting propagation, successful seedling establishment depends on coordinated root–shoot development, rather than on isolated root overgrowth. Root morphology determines the spatial extent over which the plants can explore the substrate for water and nutrients, whereas root physiological capacity determines how effectively this structural capacity is translated into actual resource acquisition [51]. The four rooting promoters were found to target different aspects of root development. *Bacillus amyloliquefaciens* primarily promoted root elongation and lateral root formation, whereas 5-ALA favored root-

system expansion while simultaneously enhancing root physiological activity and cutting survival. GABA, by contrast, supported a more balanced development of belowground roots and aboveground shoots. Previous studies have shown that low concentrations of 5-ALA can alter root elongation by modulating polar auxin transport [46], which may partly explain the pronounced root-volume-related responses observed under the 5-ALA treatment in the present study. Some *B. amyloliquefaciens* strains have also been reported to reshape lateral root and root-hair development through auxin- and cytokinin-related processes [49], which is consistent with the highly branched root phenotype observed under the *B. amyloliquefaciens* treatment. However, these mechanisms have mainly been demonstrated in other plant systems and have not yet been verified in tea chrysanthemum. Collectively, our results indicate that greater root abundance alone cannot guarantee high-quality seedlings, and root physiological activity is equally critical, as corroborated by the metabolic and antioxidant responses measured in this study.

Root development is a prerequisite for successful cutting establishment, whereas the internal metabolic balance directly influences the sustained physiological activity of the roots. Soluble metabolites and antioxidant-related traits provide useful indicators of this physiological state. The marked differences in soluble sugar and soluble protein contents among the treatments suggest that the rooting promoters altered carbon and nitrogen allocation rather than simply increasing the accumulation of osmotic solutes. The broad metabolic effects of GABA may be explained, at least in part, by the GABA shunt, which connects GABA metabolism with the tricarboxylic acid cycle and is closely coupled with carbon and nitrogen metabolism [52–54]. A similar homeostatic pattern was evident in the antioxidant system. The treatment showing the lowest oxidative damage did not exhibit the highest activity of all the antioxidant enzymes. Reactive oxygen species have dual roles as developmental signals and potential sources of cellular damage; therefore, plant performance depends on maintaining a dynamic balance between ROS production and scavenging. The lower H₂O₂ accumulation and lipid peroxidation observed under the GABA treatment are therefore more consistent with a more stable whole-plant redox status than with the nonspecific activation of all the antioxidant enzymes. Studies in several crop species have likewise shown that exogenous GABA can improve metabolic adjustment and stress tolerance, while a stable redox balance helps protect both root function and photosynthetic tissues and thereby supports sustained seedling growth [55–57].

Plant metabolic status is ultimately reflected in photosynthetic productivity, and the four rooting promoters appeared to regulate the photosynthetic system through distinct physiological routes. Chlorophylls and carotenoids contribute to light harvesting and photoprotection, whereas realized carbon assimilation is additionally influenced by stomatal conductance, CO₂ diffusion, leaf water status, biochemical fixation capacity, respiration, and sink demand [58,59]. Therefore, the treatment showing the greatest pigment accumulation does not necessarily exhibit the highest carbon assimilation efficiency. The stronger pigment response under the *Bacillus amyloliquefaciens* treatment may have been indirectly supported by its more developed root system, which could enhance water and nutrient acquisition and thereby help maintain photosynthetic apparatus integrity. In other plant systems, *B. amyloliquefaciens* inoculation has been shown to increase root biomass and root activity, together with plant P status and chlorophyll accumulation [60]. In contrast, GABA was associated with a more favorable gas-exchange performance, suggesting a broader effect on whole-plant metabolic regulation. Previous studies have shown that GABA can regulate ALMT-type anion transporters, thereby linking intracellular signaling with membrane transport and root growth [47]; however, ALMT activity was not measured in the present study, and this pathway therefore cannot be directly verified here. 5-Aminolevulinic acid (5-ALA) is a precursor of tetrapyrrole biosynthesis, including the

chlorophyll branch [44], and exogenous ALA has been experimentally shown to increase chlorophyll accumulation at appropriate concentrations [61]. This metabolic connection may therefore contribute to the pigment response observed under 5-ALA treatment.

The rhizosphere substrate is an active component of root–shoot performance, and studies in chrysanthemum have shown that changes in substrate physicochemical properties, nutrient status, rhizosphere activity, and root-associated processes are closely linked to plant performance [62]. In the present study, the four rooting promoters produced distinct changes in the substrate physicochemical and biochemical properties. The total N, P, and K were interpreted as bulk nutrient pools, whereas the available N, P, and K represented the more labile nutrient fractions potentially accessible for plant acquisition. The urease, phosphatase, and sucrase activities can be used as biochemical indicators associated with N, P, and C cycling, respectively, but a higher enzyme activity does not by itself demonstrate a greater nutrient uptake by the plants [63]. *Bacillus velezensis* altered the rhizosphere environment. This response is consistent with the experimental evidence that some *B. velezensis* strains secrete low-molecular-weight organic acids, decrease the pH of their surrounding medium, and promote the solubilization of mineral K and P [64].

Considering the full set of plant morphological, physiological, photosynthetic, rhizosphere, and other measured traits, the four root-promoting agents exhibited four distinct regulatory strategies across the substrate–root–shoot system. 5-ALA predominantly promoted root establishment and physiological activity; *Bacillus amyloliquefaciens* mainly acted by reshaping the root architecture; *Bacillus velezensis* primarily altered the rhizosphere biochemical environment; and GABA showed no single dominant regulatory target but instead simultaneously enhanced multiple processes, including root function, photosynthetic carbon assimilation, metabolic balance, antioxidant capacity, and rhizosphere nutrient supply. The value of multivariate analysis and comprehensive scoring lies not merely in grouping and ranking the treatments but also in demonstrating that high-quality seedlings cannot be achieved through the enhancement of a single trait alone; rather, coordination among multiple physiological processes is critical. Thus, the principal advantage of GABA lies in its coordinated regulation across multiple systems rather than in the enhancement of any single trait. This also explains why some of the treatments performed particularly well for individual indicators but resulted in lower overall seedling quality than GABA.

This study has several limitations. All the cuttings were subjected to a uniform IBA pretreatment, and no blank control without IBA treatment was included. In addition, only a single concentration was tested for each root-promoting treatment. The experiment was conducted exclusively under plateau conditions, and rhizosphere colonization by the bacterial strains was not verified; therefore, the dose–response relationships and the potential effects of the altitude-related environmental conditions could not be comparatively evaluated. Furthermore, endogenous hormones, key metabolic intermediates, root exudates, and actual plant nutrient uptake were not quantified. Consequently, the present study can only demonstrate that different root-promoting agents induce distinct root–shoot–rhizosphere responses but cannot yet establish the precise causal mechanisms underlying these phenotypic differences. Future studies should build on these findings to further elucidate the distinct molecular regulatory mechanisms of the four root-promoting agents and, in particular, clarify the mechanisms underlying the coordinated multi-system regulation mediated by GABA.

5. Conclusions

This study demonstrated that all the chemical and microbial rooting promoters further improved the growth and the plateau environmental adaptability of tea chrysanthemum cuttings and alleviated the plateau growth inhibition relative to the sole IBA-pretreated

control, with distinct functional differences among the treatments. The 5-ALA treatment (T1) excelled in improving rooting and seedling survival by enhancing the antioxidant capacity. The *Bacillus amyloliquefaciens* treatment (T3) mainly promoted root elongation and optimized rhizosphere nutrient conditions, while *Bacillus velezensis* (T4) only produced minor improvements in seedling performance. By comparison, the GABA treatment (T2) comprehensively optimized the photosynthetic physiology, the antioxidant stability and the rhizosphere microenvironment, coordinately facilitating root and shoot growth. Consistently, a multi-index D-value evaluation confirmed T2 had the best overall performance among the tested treatments, with the ranking of T2 (0.83) > T3 (0.80) > T1 (0.67) > T4 (0.62) > CK (0.11). These findings provide theoretical and technical guidance for the differentiated application of diverse rooting promoters in plateau tea chrysanthemum cutting propagation. Future studies will conduct concentration gradient and multi-environment tests to further improve the general applicability of these rooting promoters for tea chrysanthemum seedling production on plateaus.

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