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Moderate Boron Concentration Beneficial for Flue-Cured Tobacco (*Nicotiana tabacum* L.) Seedlings Growth and Development

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Abstract: Boron (B) deficiency is a common phenomenon in most tobacco-planting areas in Yunnan, China. In 2020 and 2022, hydroponic experiments that contained B in a concentration gradient of 0.000, 0.125, 0.250, 0.750, 5.000, 10.000, 20.000, and 40.000 mmol L^{-1} were conducted to investigate tobacco cultivar K326′s agronomic traits, photosynthetic performance, antioxidant enzymes, and boron and nicotine concentration. As B concentration increased, indices including leaves biomass and net photosynthetic rate (Pn) generally increased first and then decreased, which was in contrast to antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). With increasing B concentration, boron content in tobacco seedlings increased significantly by 24.00~96.44%, while decreased nicotine content by 21.60~82.03%. The highest biomass and photosynthetic performance were obtained within 0.75 and 5.00 mmol L^{-1} treatments. The results of the sandy soil pot verification experiment were similar to the hydroponic experiment obtained. The beneficial mechanism of moderate B on tobacco seedlings is to maintain cell structure integrity, enhance photosynthetic capability, and promote root growth. Consequently, the optimum B concentration for tobacco seedlings is 0.75~5.00 mmol L^{-1}, and applying 0.25~0.50 B kg hm^{-1} in soil under available B insufficiency could meet the needs of the growth of flue-cured tobacco.

Keywords: micronutrient; photosynthesis; physiology and biochemistry; enzyme activity; nicotine

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1. Introduction

Boron (B) is an indispensable nutrient for the growth of plants, and it is relevant in physical and metabolic functions, including synthesis of plant uracil nucleochlorophyll, cytokinins, auxin, promote root development, cell-wall pectin formation [1,2], protein metabolism, alkaloid production, etc., thus affecting the growth and development of plants [3]. Boric acid (BO_3^{3-}) is the main form of absorption of B by plants. Tobacco (*Nicotiana tabacum* L.) is an important cash crop and pillar industry in China, and the total national revenue of the tobacco industry reached USD 200 billion in 2020, playing an important role in supporting the national fiscal revenue. Tobacco is sensitive to B and the response curve is reported to have a very narrow sufficiency range, which means that even in B-deficient tobacco planting areas, excessive B supplementation is likely to cause malnutrition of tobacco plants [4,5] Appropriate concentration of B increases root system, leaf area, photosynthetic capacity, and dry matter accumulation of tobacco plants, and improves stress resistance, thus laying a good substance foundation for the growth of flue-cured tobacco in the field. Therefore, exploring an optimum B application rate for

major cultivar K326 of flue-cured tobacco seedlings is crucial for achieving sustainable development and production of tobacco.

Plants under B deficiency condition generally show growth retardation, limitation of the elongation and expansion of leaves, deformation of the leaves, and cessation of the bud [6,7]. Additionally, boron deficiency leads to the production of large amounts of auxin, thus prompting branches of root then increasing nicotine content [8]. Plants' physiological function and metabolism are also disturbed under B deficiency condition [9]. Specifically, increasing cell membrane permeability and the accumulation of reactive oxygen species would damage the normal structure and function of cells [10]. Boron toxicity, on the other hand, causes a great loss of crop yield worldwide. Previous studies have shown that when solution B concentration was at or above 200 mol/L, the tobacco plant showed B poisoning symptoms [11]. Plants with B toxicity are usually characterized by leaf vein loss of greenness, leaf tip burn, leaf tip curling, and necrotic patches at the tips or margins of older leaves [12]. As for tobacco, the B poisoning symptoms were characterized by brown spots at the tips or edges of the old leaves, which then developed towards the midrib and base. In some extreme cases, the brittleness of the lamina and rapid foliation could be recorded [13]. Barley leaf length, width, and area do not show any differences between excessive and normal B application. However, root weakness and death of lateral root were noted in the cultivation of excessive solution B concentration [14,15]. In addition, oxidative stress becomes severe under B poisoning, manifested by significantly elevated superoxide dismutase (SOD), peroxidase (POD), ascorbic acid peroxidase (APX), and catalase (CAT) activity under B overdose conditions [16–18].

There are many studies that have investigated the effects of B deficiency on yield, quality, and physiological and biochemical characteristics of flue-cured tobacco in the field stage [19–21]; however, there are limited reports on B on photosynthesis characteristics, antioxidant enzymes, and nicotine content in the seedling stage. By using a combination of hydroponic experiment and sandy soil pot experiment, the objectives of this study were to identify the critical values of B deficiency and toxicity in tobacco seedlings, to explore the optimal concentration of B in actual production, to provide a basis for the regulation of B nutrition in the production, and, further, to enrich the physiological mechanism of B on flue-cured tobacco, which is of great significance for the yield and quality of flue-cured tobacco.

2. Materials and Methods

2.1. Experiment Design

This study was carried out at the greenhouse of Yunnan Academy of Tobacco Agricultural Science, Yuxi, China (24°14′ N, 102°30′ W; altitude 1680 m) in 2020 and 2022. On 18 September 2020 (6 weeks after germination), uniform tobacco seedlings (variety K326, a most conventional commercial tobacco cultivar worldwide) with a single stem were selected to transplant into 500 mL pots containing $\frac{1}{2}$ B-free Hoagland's solution. The pH was adjusted to 6.0 with 0.1 mol/L NaOH and HCl. The growing condition was kept at 25 °C under 16 h light period, humidity about 75%, and average irradiation 650 $\mu M \cdot m^{-2}\ s^{-1}$. The roots were covered with tin foil to protect them from light to promote root growth and were ventilated for 2 h a day, and the nutrient solution was replaced within the interval of 3 days. After 7 days of preculture, eight different B concentrations (H_3BO_3) were used (0.00 (B1), 0.125 (B2), 0.25 (B3), 0.75 (B4), 5.00 (B5), 10.00 (B6), 20.00 (B7), and 40.00 (B8) $mmol \cdot L^{-1}$) to induce B deficiency and toxicity [4,8,11]. The nutrient solution without BO_3^{3-} (0.00 $mmol \cdot L^{-1}$) was set as the control. Each treatment was repeated three times and the biochemical analyses were conducted 30 days after treatment application.

On 10 January 2022 (6 weeks after germination), the uniform tobacco seedlings (varieties K326) with four leaves were selected and transplanted into a pot with sandy soil. The sandy soil was sterilized and dewormed with carbendazim and trichlorfon in advance. After 7 days of preculture, eight different B concentrations were conducted at 0.00, 0.25, 0.50, 0.75, 1.00, 2.00, 4.00, and 10.00 $B \cdot kg \cdot hm^{-2}$, according to the planting of 15,000 tobacco

plants per hectare. Numerically the weight of H_3BO_3 of 0.00 g (CK-S), 0.0167 g (B1-S), 0.0333 g (B2-S), 0.0500 g (B3-S), 0.0833 g (B4-S), 0.1333 g (B5-S), 0.2667 g (B6-S), and 0.5333 g (B7-S) were applied to each tobacco seedling under different treatments, respectively. Other nutrients were replenished by B-free Hoagland solution, and the B-free Hoagland solution was applied once every 3 days for 2 L each. Each pot was transplanted with one plant, and the positions of the tobacco seedlings were randomly adjusted to ensure more consistent growth and illumination conditions for each tobacco. During the experiment, the day length was 14 h, temperature kept at 25 °C, humidity maintained at 75%, and light intensity was 650 $\mu M \cdot m^{-2}\ s^{-1}$. Relevant physiological indexes were sampled and measured when the deficiency or toxicity symptoms were shown in this study. The basic nutrients of the trail soil are shown in Table 1.

Table 1. Basic properties of the trail soil.

Soil Type	Texture	Cation Exchange Capacity $cmol \cdot kg^{-1}$	Organic Matter $mg \cdot kg^{-1}$	Total N $mg \cdot kg^{-1}$	Total P $mg \cdot kg^{-1}$	Total K $mg \cdot kg^{-1}$	Available B $mg \cdot kg^{-1}$
Sandy soil	Sand	1.26	3.65	0.14	0.07	23.75	0.167

2.2. Analyses Methods

2.2.1. Agronomic Traits

Under hydroponic experiment, 30 days after transplanting, the height of tobacco seedlings was determined from the ground along the stem to the growing tip, and the leaf length and width were determined by the sixth leaf from the leaf base to the growing tip.

2.2.2. Biomass of Tobacco Seedlings

After being killed at 105 °C for 10 min and dried at 55 °C to the constant weight, the root, stem, and leaf dry weight were measured and calculated to obtain the total weight.

2.2.3. Soil–Plant Analysis Development (SPAD) Value

Chlorophyll content in tobacco seedlings leaves were measured using an SPAD-502 (Konica Minolta Inc., Tokyo, Japan) portable chlorophyll detector [22–24]. The middle part of the sixth leaf from the leaf base to the growing tip was measured three times. Three measurements were averaged as SPAD value for each observation.

2.2.4. Photosynthetic Parameters

At the end of the experiment, the photosynthetic parameters, such as the photosynthetic rate (Pn; $\mu mol \cdot m^{-2} \cdot s^{-1}$), stomatal conductance (Cond; $mol \cdot m^{-2} \cdot s^{-1}$), intercellular CO_2 concentration (Ci; $\mu mol \cdot mol^{-1}$), and transpiration rate (Tr; $mmol \cdot {}^{-2} \cdot s^{-1}$), of all plants were measured using the Li-6400 portable photosynthetic apparatus (Li-COR Inc., Lincoln, NE, USA). Before the measurement, the leaf chamber photosynthetic active radiation intensity (PAR) was set to 1800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to induce the leaves. During the measurement, the set value of the CO_2 injection system was 400 $\mu mol \cdot mol^{-1}$, the gas flow rate was 500 $mmol \cdot s^{-1}$, the leaf temperature was 25 °C, and the leaf chamber PAR was set to 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Finally, the automatic measurement procedure was started when the photosynthesiometer readings were stable. The average value of the three measurements was taken to facilitate the analysis.

2.2.5. Boron Ion Content in Tobacco Seedlings

Boron concentration was determined according to the wet ashing technique (10 mL nitric and 5 mL of 70% perchloric acid digestion method), and the atomic absorption spectrophotometry measurements were used in the determination [25].

2.2.6. Nicotine Concentration

The nicotine concentration of tobacco leaves was measured by gas chromatography with minor modification [26]. Briefly, 0.1 g tobacco leaf sample was freeze-dried and ground into fine powder. After soaking in 1 mL of 10% (*w*/*v*) NaOH for 20 min, the sample was extracted in the equivalent volume of dichloromethane with vortexing. The nicotine content was determined using an Agilent Technologies 7890 A Chromatograph equipped with a DB 5 MS column, using nicotine from Sigma-Aldrich as a standard control. The data for each observation were determined from three independent replicates.

2.2.7. Defense-Related Enzymes and Compounds

A total of 0.5 g tobacco seedling leaves with uniform symptoms of B deficiency or B poisoning were taken to determine the activity of antioxidant enzymes. SOD activity was evaluated by SOD kit (NBT method) [27], POD activity was accessed by POD kit [28], CAT activity was checked by CAT kit (UV absorption method) [29], and ascorbate peroxidase (APX) activity was evaluated by APX kit (UV absorption method) [30]. These applying kits were provided by Keming Biotechnology Co., Ltd., Suzhou, China.

2.2.8. Oxidative Stress Indicators

Malondialdehyde (MDA) content was determined by MDA kit [31], and H_2O_2 content was accessed by H_2O_2 kit [32]. These applying kits were provided by Keming Biotechnology Co., Ltd., Suzhou, China.

2.2.9. Nitrate Reductase (NR) Activity

Nitrate reductase (NR) was determined by NR kit [33], and this determination kit (UV absorption method) was procured from Keming Biotechnology Co., Ltd., Suzhou, China.

2.2.10. Soil Physical and Chemical Parameters

Before the experiment was conducted, 500 g air-dried soil samples were used to perform routine analysis for texture, cation exchange capacity, soil organic matter, soil total nitrogen, phosphorus, potassium, and soil available boron.

Soil cation exchange capacity was determined via potentiometric titration [34]. Soil texture (clay, silt, and sand) was measured by hydrometric method [35]. Soil organic matter was determined by using the potassium dichromate oxidation method [36]. Soil total nitrogen was determined by using a micro-Kjeldahl digestion followed by steam distillation, total phosphorus was quantified using the molybdenum–antimony antispectrophotometric method, and total potassium was measured by flame photometry [37]. Soil available boron concentration in sandy soil was determined according to the curcumin–oxalic acid method [38]. The concentration of available B in the sandy soil before the pot experiment was 0.167 mg/kg. The B ion concentrations in sandy soil of each treatment were 0.213, 1.704, 2.716, 3.507, 6.679, 7.634, 11.953, and 22.829 mg/kg, respectively.

2.3. Statistical Analysis

Data were statistically analyzed by the single factor analysis of variance (one-way ANOVA) and Spearman correlation in SPSS 20.0 (IBM SPSS Statistics Inc., Chicago, IL, USA; SPSS Inc., Chicago, IL, USA). Triplicate measurements on tobacco plants samples (n = 3) were averaged for statistical analysis of treatments effects. Treatment effects were considered significant at alpha equals 0.05 level of probability. Means separation was performed using Duncan's multiple test. Figures were created with GraphPad Prism 8 Software (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Effect of B Solution Concentration on Seedling Growth and Shoot Biomass Formation

The growths of flue-cured tobacco seedlings treated with different B concentrations after 30 days of hydroponics condition are shown in Figure 1. The 0.00 mmol·L^{-1} treatment

showed B deficiency symptoms with light green color, corking of leaf veins, and leaf marginal curling. With B concentration in solution increased from 5.00 mmol·L^{-1} to 40.00 mmol·L^{-1}, the area of scorched leaf surface gradually expanded from leaf tip to whole leaf as per tobacco B toxicity symptoms. At 40.00 mmol L^{-1} treatment, the lower leaves of flue-cured tobacco were completely withered to deformation.

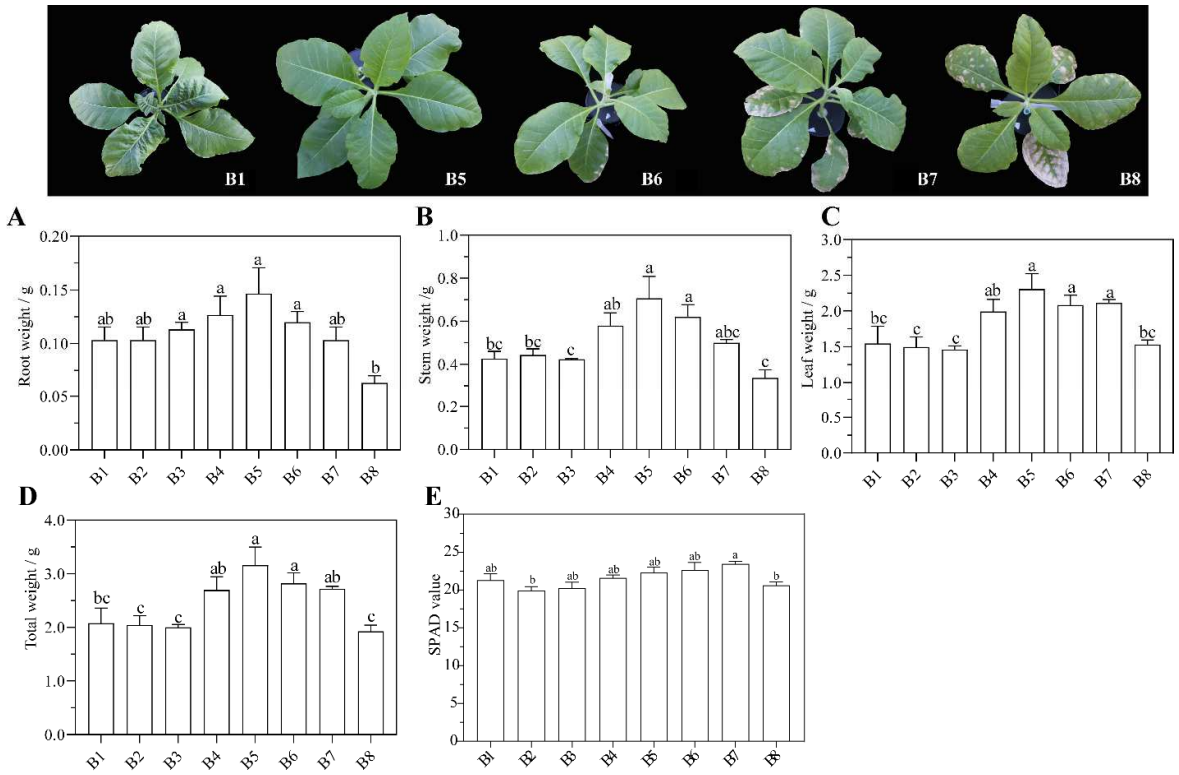

Figure 1. Effects of B on the growth performance of tobacco seedlings. Note: growth statuses of flue-cured tobacco seedlings under different B concentrations: 0.00 mmol·L^{-1} (B1), 5.00 mmol·L^{-1} (B5), 10.00 mmol·L^{-1} (B6), 20.00 mmol·L^{-1} (B7), 40.00 mmol·L^{-1} (B8). B1 treatment showed B deficiency symptoms, B5 showed a normal growth condition, and B6 to B8 showed B toxicity symptoms. (**A**) Root dry weight. (**B**) Stem dry weight. (**C**) Leaves dry weight. (**D**) Total weight. (**E**) SPAD value. Data are means ± standard errors (n = 3). Within a column, values followed by different lower letters are significantly different at $p < 0.05$ following Duncan's multiple-range test.

All plant growth parameters were significantly different between the eight treatments ($p < 0.05$) (Figure 1A–D). With the increase in B concentration, tobacco seedlings' leaf, stem, root, and total dry weight showed a downward parabolic trend, and peaked at B concentrations at B5 treatment, which, respectively, increased by 9.68~36.56% (leaf), 12.26~52.36% (stem), 13.64~56.82% (root), and 10.65~38.92% (whole plant), compared with other treatments.

The B concentration in solution did not affect the plant height and stalk width (Figure 2A,B), while it significantly ($p < 0.05$) affected the leaf number (Figure 2C), maximum leaf length (Figure 2D), and leaf width (Figure 2E). When B concentration was at B4 treatment, leaf length increased 4.83~20.175%. At B5 treatment, the leaf width was higher than other treatments by 0.77~24.36%. The leaf number peaked at B7 treatment, and was significantly higher than those of B2 and B8, compared with other treatments, which increased by 2.38~19.05%.

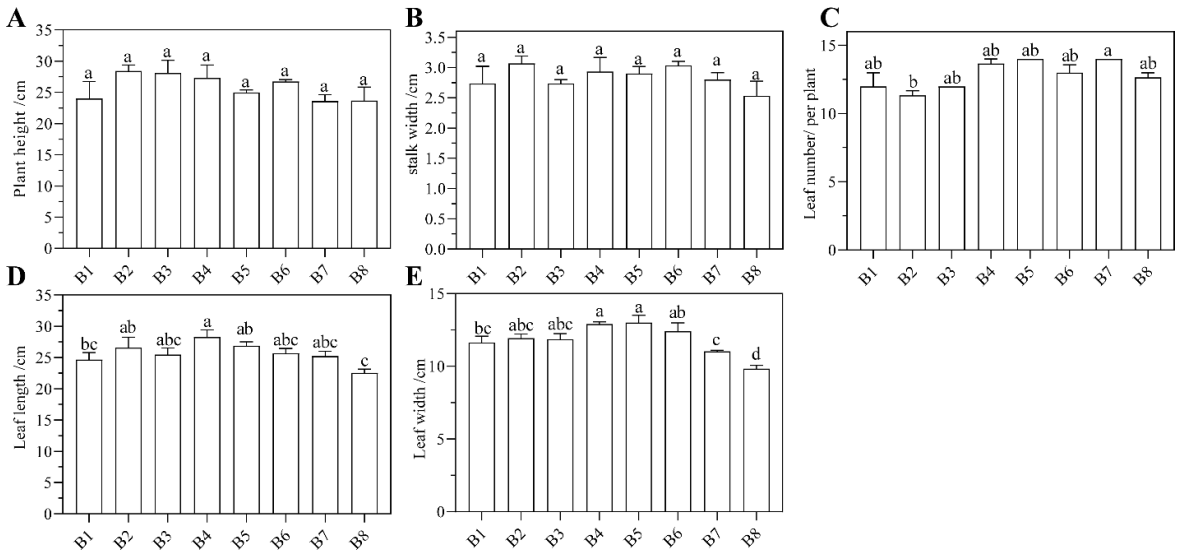

Figure 2. Characteristics of agronomic parameters of flue-cured tobacco under different B concentrations. Note: tobacco characteristics of agronomic parameters of flue-cured tobacco under different B concentrations. (**A**) Plant heights. (**B**) Stalk width. (**C**) Leaf number. (**D**) Leaf length. (**E**) Leaf width. Data are means ± standard errors (n = 3). Within a column, values followed by different lower letters are significantly different at $p < 0.05$ following Duncan's multiple-range test.

3.2. Effect of B Solution Concentration on Photosynthetic Performance

Boron concentrations significantly impacted the SPAD value of flue-cured tobacco seedlings leaves ($p < 0.05$) (Figure 1E). SPAD value reached its maximum at B7, which increased by 9.16% (B1), 15.04% (B2), 13.71% (B3), 7.88% (B4), 4.84% (B5), 3.46% (B6), and 12.00% (B8), respectively.

Photosynthetic properties of tobacco seedlings were significantly influenced by solution B concentration ($p < 0.05$; Table 2). Photosynthetic properties parameters showed a parabolic trend with the increase of B concentration. The Pn parameters peaked at B4 treatment and were significantly increased by 63.96%, 46.83%, and 80.28% compared with B1, B7, and B8, respectively. The Cond parameter at B4 increased the most, by 33.40%~75.86%, compared to other treatments. The Ci parameters reached the maximum at B3 treatment, which was 0.83%~57.95% higher than other treatments. The Tr parameters reached the highest at B3 treatment, which increased by 49.02% (B1), 30.83% (B2), 62.05% (B7), and 61.55% (B8), respectively.

Table 2. Photosynthetic rates of tobacco seedlings under different B application rates.

Treatments	Pn/(μmol/(m^2·s))	Cond/(mol/(m^2·s))	Ci/(μmol/mol)	Tr/(mmol/(m^2·s))
B1(CK)	4.29 ± 1.43 cd	0.064 ± 0.011 cd	397.77 ± 6.26 b	2.56 ± 1.15 cd
B2	10.25 ± 1.12 a	0.080 ± 0.025 cd	497.29 ± 4.83 a	3.48 ± 0.68 bc
B3	10.50 ± 1.50 a	0.108 ± 0.025 bc	501.48 ± 5.95 a	5.02 ± 0.82 a
B4	11.90 ± 1.59 a	0.205 ± 0.038 a	410.79 ± 8.05 b	4.75 ± 0.70 ab
B5	11.75 ± 1.62 a	0.129 ± 0.027 b	267.78 ± 4.46 c	3.70 ± 0.88 abc
B6	9.61 ± 1.32 ab	0.136 ± 0.021 b	381.16 ± 4.5 b	3.79 ± 0.82 abc
B7	6.33 ± 1.59 bc	0.071 ± 0.020 cd	254.11 ± 7.04 c	1.91 ± 0.64 d
B8	2.35 ± 0.74 d	0.049 ± 0.017 d	210.85 ± 6.29 c	1.93 ± 0.78 d

Note: Data are means ± standard errors (n = 3). Different lower letters denote significant differences ($p < 0.05$ following Duncan's multiple-range test).

3.3. Effect of B Solution Concentration on Antioxidant Enzymes and MDA Content

Antioxidant enzymes in tobacco seedlings were significantly influenced by B ($p < 0.05$, Figure 3). As solution B concentration increased, the SOD, POD, and CAT activities showed a "down-up" trend. SOD activities reached a maximum at B7, by 10.44~91.67%,

compared to other treatments. POD activity was increased more severely at B6 treatment, by 21.32~65.62%, than other treatments. When B concentration was at B7 treatment, the CAT activities were significantly higher than other treatments, increasing by 50.05~96.44%. Concentrations at B4 had minimal MDA content with significant variability between each treatment ($p < 0.05$), which decreased by 46.65~302.57%.

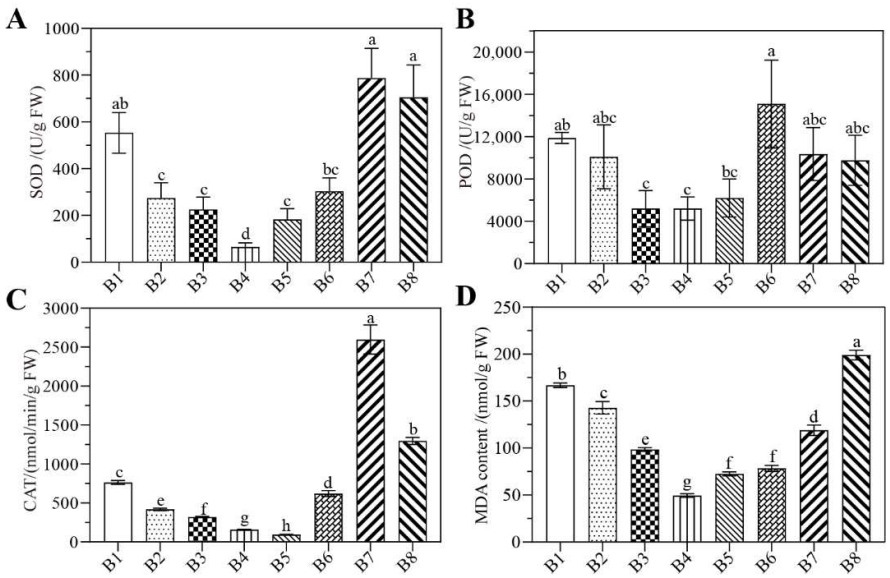

Figure 3. Antioxidase activities and MDA content of flue-tobacco seedlings under different B concentrations. Note: antioxidant enzymes activities and MDA content in flue-cured tobacco seedlings under different B concentrations. (**A**) SOD activities. (**B**) POD activities. (**C**) CAT activities. (**D**) MDA content. Different lower letters denote significant differences at $p < 0.05$ following Duncan's multiple-range test.

3.4. Effect of B Solution Concentration on B and Nicotine Concentration in Plant Tissue

Boron concentrations in tobacco seedlings' root, stem, and leaf were significantly affected by solution B concentration ($p < 0.05$, Figure 4A). With the increase of solution B concentration, there were sharp increases in the root, stem, and leaf tissue. All parameters were significantly different and reached the maximum at B8 treatment. Compared with B8 treatment, the concentration of B ion accumulation increased by 24.43~86.76% (roots), 26.97~96.44% (stems), and 24.00~67.01% (leaves), respectively.

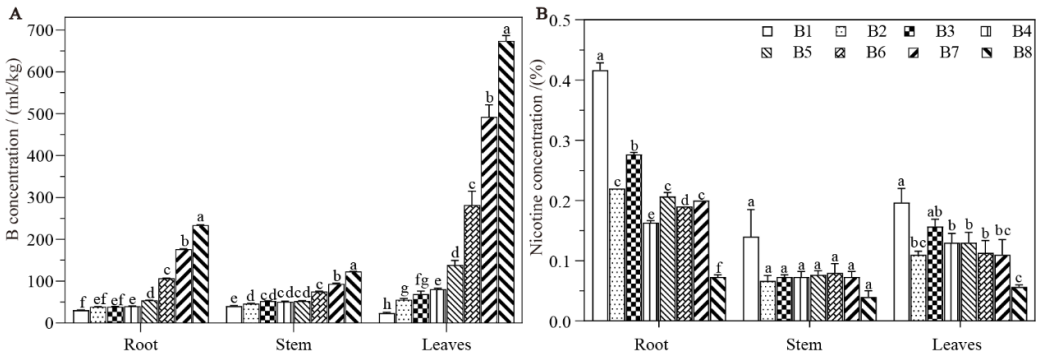

Figure 4. Changes of B and nicotine concentration in different parts of flue-cured tobacco under different B concentrations. Note: concentration of B and nicotine content in each part of tobacco seedlings under different B concentrations. (**A**) B concentration. (**B**) Nicotine concentration. Data are means ± standard errors ($n = 3$). Within a column, values followed by different lower letters are significantly different at $p < 0.05$ following Duncan's multiple-range test.

The concentration of nicotine in roots and leaves of tobacco seedlings was significantly affected by B in solution ($p < 0.05$, Figure 4B). Nicotine concentration in root and leaves declined significantly when B concentration was at or above B1 treatment; however, the nicotine content in stems did not show significant difference among treatments. Compared with other treatments, B1 was increased by 33.58~82.03% (roots), 43.83~72.93% (stems), and 21.60~70.34% (leaves), respectively.

3.5. Indices Related to Different B Solution Concentration

The correlation analysis shows that the different B solution concentration was positively correlated with the accumulation of B ion in each part of tobacco seedlings ($p < 0.01$), and the correlation coefficients were 0.976 (roots), 0.943 (stems), and 0.985 (leaves), respectively (Table 3), while the nicotine content in roots and leaves of flue-cured tobacco seedlings was negatively correlated with the different B solution concentration ($p < 0.01$), and the correlation coefficients were −0.783 (roots) and −0.622 (leaves).

Moreover, the accumulation of B ion in each part of tobacco seedlings were negatively correlated with the nicotine content in each part of tobacco seedlings, and the correlation coefficients varied from −0.756 to −0.476 ($p < 0.01$). The activities of SOD and CAT and the content of MDA were negatively correlated with the Pn and Cond parameters, and the correlation coefficients varied from −0.869 to −0.696 ($p < 0.01$). The correlation of Ci parameter between the accumulation of B concentration in tobacco seedlings was negative, and the correlation coefficients varied from −0.709 to −0.618 ($p < 0.01$).

3.6. Effect of B Concentration on Plant Growth and Physiological Indices under Pot Experiment

The pot experiments were designed to address the inappropriate use of B in the field and verification of the results concluded under the hydroponics condition. The concentration of B in sandy soil before the pot experiment was 0.167 mg·kg^{-1}. A total of 15 days after application of different B concentrations, the B deficiency symptoms were observed at B1-S treatments with leaf marginal curling, while the B toxicity symptoms were observed at B3-S, B4-S, B5-S, B6-S, and B7-S treatments with scorched appearance, extreme stunting, and even plant death (Figure 5). With the increase of B concentration in sandy soil, the leaves withered, and experienced thinness, stunting, and complete death. However, these hydroponics that had B deficiency symptoms did not show any symptoms in the pot environment.

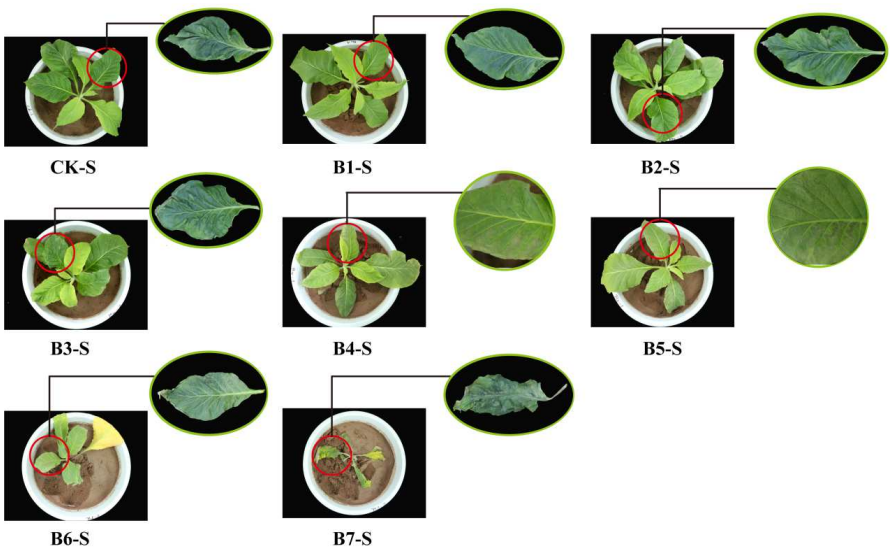

Figure 5. Growth statuses of flue-cured tobacco seedlings in pot experiment under different B concentrations. Note: 0.00 B kg·hm^{-2} (CK-S), 0.25 B kg·hm^{-2} (B1-S), 0.50 B kg·hm^{-2} (B2-S), 0.75 B kg·hm^{-2} (B3-S), 1.00 B kg·hm^{-2} (B4-S), 2.00 B kg·hm^{-2} (B5-S), 4.00 B kg·hm^{-2} (B6-S), 8.00 B kg·hm^{-2} (B7-S).

Table 3. Spearman correlation coefficients for correlations among different B concentrations and the tobacco seedlings growth indices.

Parameter	DBC	TBR	TBS	TBL	TNR	TNS	TNL	SPAD	Pn	Cond	Ci	Tr	SOD	POD	CAT	MDA
DBC	1															
TBR	0.977 **	1														
TBS	0.943 **	0.935 **	1													
TBL	0.985 **	0.967 **	0.943 **	1												
TNR	−0.783 **	−0.756 **	−0.681 **	−0.754 **	1											
TNS	−0.490 *	−0.519 **	−0.508 *	−0.476 *	0.548 **	1										
TNL	−0.622 **	−0.636 **	−0.526 **	−0.642 **	0.629 **	0.573 **	1									
SPAD	0.321	0.291	0.238	0.32	−0.147	0.05	−0.025	1								
Pn	−0.284	−0.29	−0.298	−0.296	0.15	0.088	0.141	−0.069	1							
Cond	−0.079	−0.159	−0.098	−0.091	−0.174	0.241	0.271	0.155	0.625 **	1						
Ci	−0.733 **	−0.703 **	−0.618 **	−0.709 **	0.503 *	0.29	0.289	−0.394	0.473 *	0.262	1					
Tr	−0.36	−0.357	−0.306	−0.372	0.069	0.133	0.366	−0.326	0.563 **	0.624 **	0.557 **	1				
SOD	0.32	0.358	0.388	0.371	0.052	−0.14	−0.231	0.187	−0.696 **	−0.785 **	−0.443 *	−0.718 **	1			
POD	0.047	0.005	0.029	0.021	0.022	0.141	0.07	0.088	−0.410 *	−0.23	−0.236	−0.363	0.430 *	1		
CAT	0.378	0.391	0.421 *	0.373	−0.099	−0.116	−0.296	0.139	−0.733 **	−0.679 **	−0.351	−0.703 **	0.845 **	0.509 *	1	
MDA	−0.005	0.019	0.041	0.013	0.18	−0.129	−0.217	−0.243	−0.705 **	−0.869 **	−0.167	−0.651 **	0.715 **	0.382	0.714 **	1

Note: DBC = different B concentration; TBR = total B content in root; TBS = total B content in stem; TBL= total B content in leaves; TNR = total nicotine content in root; TNS = total nicotine content in stem; TNL = total nicotine content. * Correlation was at significant level ($p < 0.05$); ** correlation was at extremely significant level ($p < 0.01$).

Under pot experiments (Figure 6), SOD and POD activities reached a maximum at B6-S treatment, by 18.36~168.65% (SOD) and 45.39~965.44% (POD) compared to other treatments, respectively (Figure 6A,B). Under APX index, B5-S reached the highest, which increased by 25.26 times (CK-S), 11.12 times (B1-S), 6.37 times (B2-S), 76.85% (B3-S), 35.93% (B4-S), 52.05% (B6-S), and 29.89% (B7-S), respectively (Figure 6C).

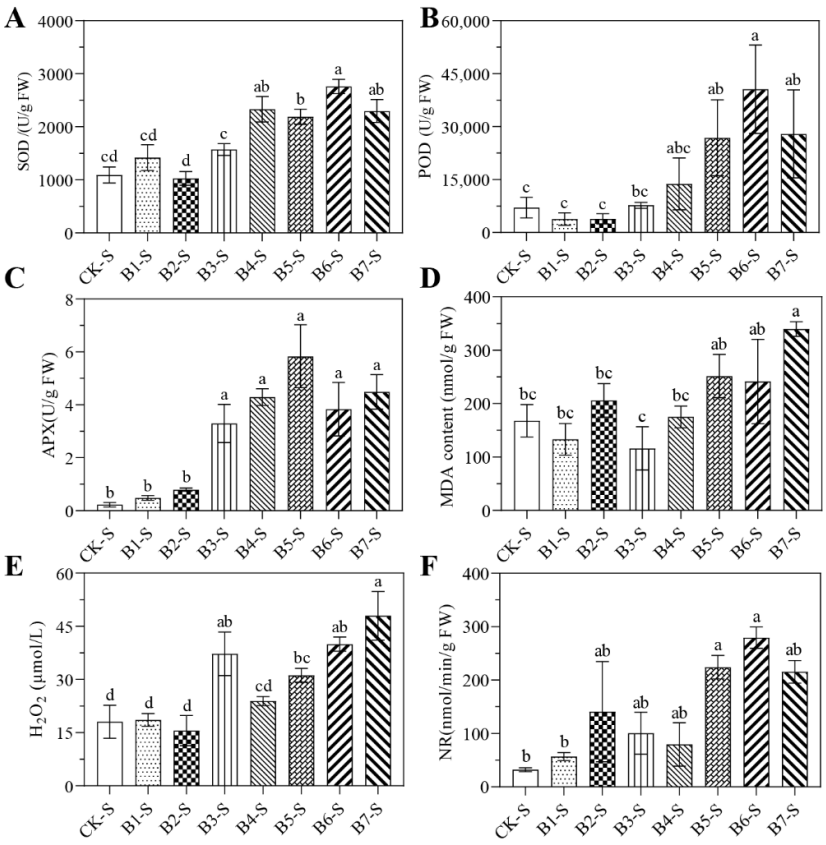

Figure 6. Physiological indices of flue-tobacco seedlings under different B concentrations. Note: physiological indices of flue-tobacco seedlings under different B concentrations. (**A**) SOD activity. (**B**) POD activity. (**C**) APX activity. (**D**) MDA content. (**E**) H_2O_2 content. (**F**) NR activity. Data are means ± standard errors (n = 3). Within a column, values followed by different lower letters are significantly different at $p < 0.05$ following Duncan's multiple-range test.

When B concentration was at B-S7 treatment, the H_2O_2 content and the MDA content were significantly higher than these of other treatments, by 20.01~207.94% (H_2O_2) and by 35.06~192.45% (MDA), respectively (Figure 6D,E).

Under the NR indicators, CK-S treatment was the lowest, which was reduced by 76.41% (B1-S), 3.38 times (B2-S), 2.13 times % (B3-S), 1.47 times % (B4-S), 5.97 times (B5-S), 7.69 times % (B6-S), and 5.70 times % (B7-S), respectively (Figure 6F).

4. Discussion

4.1. Effect of B Solution Concentration on Tobacco Seedling Agronomic Traits, and Photosynthetic Performance under Hydroponic Condition

The yield and quality of tobacco leaves are core targets for tobacco production and growers, and leaf length, leaf width, and photosynthesis are the basis of yield and quality formation. Boron is involved in ions transport and maintains the photosynthetic area, transpiration rate, and stomatal conductance in plants [39]. Therefore, moderate B concentration improves photosynthesis ability and ultimately improves the accumulation of dry matter in plants [20,40]. This finding is that with the increase of B concentration, the leaf, root, stem, and total dry matter mass of tobacco seedlings showed a unimodal curve change,

and when the concentration was 5.00 mmol L^{-1}, all parameters reached the maximum. However, in terms of photosynthesis ability, when B concentration was at 0.75 mmol L^{-1}, net photosynthetic rate (Pn) and stomatal conductance (Cond) reached a maximum, while those of both intercellular CO_2 concentration (Ci) and transpiration rate (Tr) would reach maximum at 0.25 mmol L^{-1}. These results implied that tobacco plants subjected to a severe B deficiency (>0.25 mmol L^{-1}) or excess (<5.00 mmol L^{-1}) had significant inhibition of photosynthesis ability, leading to insufficient energy, and suppressing the accumulation of plant matter. Moreover, previous studies indicated that if Ci is consistent with the change in Pn and Cond, then the decrease in Pn could be considered as being limited by stomata factors. Otherwise, it is restricted by nonstomatal factors [41]. The results of this study showed that under different B treatments, Ci, Pn, and Cond showed a consistent trend, indicating that stomatal factors might be the major reason for the decrease in photosynthetic rate.

4.2. Effect of B Solution Concentration on Tobacco Seedling Antioxidant Enzymes and MDA Content under Hydroponic Condition

Boron plays a predominant role in the cell-wall structure of higher plants, and the primary function of this element is in cell-wall synthesis, and in maintaining its structure and integrity [20,42]. Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) are important protective enzymes for plants to resist biotic and abiotic stresses, and they mainly eliminate reactive oxygen species in cells through balanced regulation of these enzymes. Thus, the degree of damage to plants caused by stress could be accessed by antioxidant enzyme activities [43,44]. In this case, boron concentration significantly affected the antioxidant enzyme activities, and the leaves' SOD, POD, and CAT activities peaked at 20.00 mmol L^{-1}, 10.00 mmol L^{-1}, and 20.00 mmol L^{-1}, respectively. This phenomenon indicated that B toxicity damaged tobacco seedling leaves. Moreover, there was a significant difference in CAT between all treatments, indicating that CAT was more sensitive to B deficiency or toxicity stress than other enzymes.

Malondialdehyde (MDA) is one of the products of the peroxidation of membrane lipid, which could reflect the damage degree of plants by stress. In this study, when plants were treated with 0.750 mmol L^{-1}, the MDA content was significantly reduced. These results suggest that a certain concentration of B could protect the leaves from active oxygen damage, maintaining the stability of the cell structure, especially chloroplast structure, thereby increasing the activity of thylakoid photosynthetic proteins, rubiscoases, and photorespiration enzymes [45,46].

4.3. Effect of B Solution Concentration on Tobacco Seedling Nicotine Level under Hydroponic Condition

Nicotine is the most vital and the most abundant alkaloid compound in tobacco. Apart from being an important indicator of tobacco quality, it is also an important defense compound [47]. In tobacco, nicotine is synthesized mainly in tobacco roots, then is transported to other organs through the xylem [48]. In this experiment, when the B concentration was at 0.00 mmol L^{-1}, the content of nicotine in each part of flue-cured tobacco seedlings was significantly higher than that of other treatments. Some studies point out that severe B deficiency would not affect the absorption of nitrogen by flue-cured tobacco [49]. Therefore, the effect of B on nicotine would be achieved by inhibiting root elongation to induce strong branching of tobacco roots, increasing the number of fine roots and the nicotine production in roots [10]. On the contrary, boron toxicity led to a significant nicotine content decrease (Figure 4B) because an excessive supply of B (40.00 mmol L^{-1}) significantly inhibited the accumulation of root dry matter (Figure 1A). This phenomenon indicated that excessive supply of B would cause the reduced root cell division, death of apical meristems, and inhibit root biomass as well [44,50,51]. Taken together, boron affects the synthesis and accumulation of nicotine by changing the root cells' morphology and the growth of tobacco seedlings.

4.4. Interactions between Indices to Different B Solution Concentrations under Hydroponic Condition

To further clarify the effect of B on the growth and development of flue-cured tobacco seedlings, the correlation analysis of all involved indexes showed that there was a significant negative correlation between B accumulation and nicotine content in each part of flue-cured tobacco, confirming that B may influence nicotine synthesis and accumulation [52,53]. As a result, the rational use of B fertilizer can regulate the nicotine content. Furthermore, there was a significant negative correlation between photosynthetic performance (Pn, Cond, and Tr) and defense-related enzymes and compounds (SOD, CAT, and MDA). Intercellular CO_2 concentration (Ci) was significantly negatively related with B accumulation. These results indicate that the decline of photosynthetic performance was mainly due to the stomatal factors [54] and the destruction of cell structure by excessive B instead of the decrease of chlorophyll content [55]. This may be explained by the fact that excessive B would damage the plasma membrane system and increase the permeability of the leaf cell membrane, thus leading to a large number of reactive oxygen species in flue-cured tobacco seedlings [56]. Excessive reactive oxygen species could cause degreasing and peroxidation of the membrane, destroy the structure and function of biofilm, inhibit photosynthetic performance, and even cause cell death.

4.5. Preliminary Study on Production Practice

To further verify the application of the optimum B concentration in actual production, a pot experiment was designed with sandy soil as the medium. Before the experiment, the initial concentration of available B in the sandy soil was 0.167 mg/kg, and the concentration of B in the 0.00 kg/hm^2 treatment in the experiment was 0.213 mg/kg, both of which were lower than 0.45 mg/kg, indicating the lack of available B in the sandy soil [57]. A total of 15 days after the treatment, it was found that the tobacco leaves showed an obvious B poisoning phenomenon at and above the B concentration of 1.00 B kg/hm^2. In the pot experiment, with the increase in B content in the sandy soil, the activities of superoxide dismutase (SOD), peroxidase (POD), and content of malondialdehyde (MDA) were raised after a decline, which was consistent with the results of the hydroponic experiment. However, the activities of SOD and POD and the content of MDA in the pot were higher than those in hydroponics, which indicated that under pot conditions, tobacco seedlings were more susceptible to B than under hydroponics system.

Moreover, the H_2O_2 content, which is the most common reactive oxygen molecule in organisms, could damage cell membranes and accelerate the aging and disintegration of cells, while APX is the key enzyme used for metabolism of ascorbic acid, and could eliminate H_2O_2 and hydroxyl radicals [58,59]. The experimental results showed that when the B concentration in the sandy soil was over 0.50 B kg/hm^2, the APX activity was significantly increased, and reached the maximum at 2.00 B kg/hm^2. However, the H_2O_2 content generally decreased and then increased with the increasing soil B concentration. The maximum value of H_2O_2 content was reached at 8.00 B kg/hm^2, indicating that a high concentration of B damages the cell membranes and accelerates the aging and disintegration of cells.

Boron plays an important role in the process of plant nitrogen metabolism [60], and NR could directly regulate the reduction of NO_3^-, thereby regulating nitrogen metabolism and affecting photosynthetic carbon metabolism [61]. The results showed that with the increase of B concentration in the sandy soil, NR activity generally exhibited a gradual increase trend, indicating that B deficiency destroyed the homeostasis of endogenous enzymes in plants. This consequence would reduce the activity of nitrogen invertase, which was consistent with the studies of Camacho-Cristobal and Gonzalez-Fontes [49,62].

4.6. Boron Critical Value in Flue-Cured Tobacco Seedlings

Boron is an essential micronutrient for the growth of plants, and the dramatic differences in sensitivity of different plants to B concentrations may be explained by plant

species (B mobility) [63], stage of life (older plants contain more B) [64], and genotype [65]. This study observed that the B deficiency symptoms at B concentration were 0.00 mmol L^{-1} and 0.00 B kg hm^{-2}, with light green color, corking of leaf veins, and leaf marginal curling, while the B toxicity symptoms were observed from 5.00 mmol L^{-1} and 0.75 B kg hm^{-2}, with inhibition of plant growth and leaf scorch developing from the edges of the leaves, leading to necrosis (Figures 1 and 5). The deficiency symptoms may be due to the low internal concentrations of B reducing NR activity in the tobacco leaves, resulting in a decrease in leaf surface and photosynthesis, consequently leading to impaired growth and development. Excessive B would induce oxidative damage and H_2O_2 accumulation, which may contribute to the expression of the B toxicity symptoms. When B solution concentration was at 0.75 and 5.00 mmol L^{-1}, the agronomic traits and dry biomass weight were in the proper conditions, which was conducive to the growth of flue-cured tobacco seedlings.

5. Conclusions

Tobacco is a greatly important cash crop and is sensitive to B. Inappropriate B application would seriously reduce the yield and quality of tobacco leaves. When solution B concentration is beyond 0.75 to 5.00 mmol L^{-1}, the insufficient or excessive B could lead to cell structural destruction, photosynthesis inhibition, and nicotine synthesis disorder. Moreover, severe B deficiency reduced the NR activity in tobacco leaves and also resulted in photosynthesis inhibition. Notably, excessive B application significantly increased B ion accumulation but decreased nicotine content in tobacco seedlings by changing the root morphology, and inhibiting the growth and function of the root. Therefore, the optimum application amount of B in production is 0.25~0.50 B kg hm^{-2}. Overall, when soil available B concentration is lower than 0.45 mg kg^{-1}, B fertilizer of 0.25~0.50 B kg hm^{-2} should be applied to the soil before the transplanting. This study contributes to raising the knowledge on B fertilization and improves the understanding of the physiological mechanism of the interaction between B and the growth and quality of tobacco, which would be beneficial for the yield and quality of flue-cured tobacco in boron-deficient tobacco-growing areas.

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Abbreviations

SOD—superoxide dismutase; POD—peroxidase; CAT—catalase; MDA—malondialdehyde; APX—ascorbate peroxidase; NR—nitrate reductase; Pn—net photosynthetic rate; Cond—stomatal conductance; Ci—intercellular CO_2 concentration; Tr—transpiration rate; SPAD—soil and plant analysis development.

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