

# Effects of excessive nitrogen fertilizer and soil moisture deficiency on antioxidant enzyme system and osmotic adjustment in tomato seedlings

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**Abstract:** Long-term excessive application of nitrogen fertilizer induces secondary salinization of soil, which results in inhibiting plant growth. In addition, soil moisture deficiency also affects plant growth. To investigate the effects of excessive nitrogen fertilizer and soil moisture deficiency on the antioxidant enzyme system, plant water relations analyzed through pressure-volume (P-V) curve, and photosynthetic light response parameters in tomato (*Solanum lycopersicum* L. Myoko) seedlings, an indoor experiment of about 50 d was conducted using two irrigation water amounts based on field capacity (soil moisture deficiency: 50%-80%; adequate water: 70%-80%), two nitrogen fertilizer rates (moderate nitrogen; excessive nitrogen fertilizer: 0.585 g/pot) and two types of irrigation water (tap water and microbial diluent). The results showed that excessive nitrogen fertilizer (N) and soil moisture deficiency (W) reduced the biomass of tomato seedlings. In comparison to CK (combination of adequate water and tap water quality), microbial dilution (EM) increased plant biomass by 5.2%. Also, the nitrogen application increased chlorophyll relative contents (SPAD). The maximum net photosynthetic rate (Pc) decreased with nitrogen application and increased with EM application and irrigation amount. Excessive nitrogen application increased the plant nitrate reductase activity (NR). The plant NR in the N treatment showed a 13.0% increase compared to CK, and the plant NR in the treatment of nitrogen application with water deficiency (WN) increased 34.0% compared to water deficiency (W). After applying excessive nitrogen, N, EM-N, WN, EM-WN respectively increased the plant nitrate reductase activity by 13.0%, 22.9%, 34.0%, and 28.6%, compared with the corresponding treatment with moderate nitrogen (i.e., CK, EM, W and EM-W). In addition, the activities of antioxidant enzymes [superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT)] in four treatments of nitrogen application (N, EM-N, WN, EM-WN) also increased significantly. Both soil moisture and nitrogen fertilizer significantly affect the parameters of osmotic adjustment, which is manifested in the reduction of osmotic potential ( $\pi_{\text{rT}}$ ), and the increase in the osmotic concentration ( $C_{\text{osm}}$ ) and concentration difference ( $\Delta C_{\text{osm}}$ ). But the decrease in the relative water content of apoplast ( $\zeta_{\text{ap}}$ ) indicated that water deficiency and excessive nitrogen reduced the water absorption and water retention capacity of tomatoes to a certain extent. In conclusion, excessive nitrogen application and soil moisture deficiency inhibit plant growth significantly in this experiment. Meanwhile, microbial dilution can alleviate excessive nitrogen fertilizer and water stress to some extent, but the effect was not significant.

**Keywords:** antioxidant enzyme, nitrogen, osmotic adjustment, pressure-volume curve, stress, tomato, water

**DOI:** 10.25165/j.ijabe.20221502.5555

**Citation:** Zhang Y J, Yu S E, Li Z J, Chang T T, Xu Q C, Xu H L, et al. Effects of excessive nitrogen fertilizer and soil moisture deficiency on antioxidant enzyme system and osmotic adjustment in tomato seedlings. *Int J Agric & Biol Eng*, 2022; 15(2): 127–134.

## 1 Introduction

Water resource shortage is one of the most concerned issues in the whole world<sup>[1-3]</sup>. China is a country lacking water resources, and agricultural irrigation water accounts for more than 60% of China's total water consumption. Therefore, the development of deficient irrigation can improve water production efficiency and save water resources<sup>[4,5]</sup>. Vegetable production occupies an important position in China's economy. Its harvested area

occupies 45% of the world's total harvested area<sup>[6]</sup>. Since 2000, greenhouse vegetable production has increased rapidly, and it has reached 4.78 million hm<sup>2</sup> in 2018<sup>[7,8]</sup>. It accounts for 18.1% of the total vegetable and 2.1% of the total agricultural area<sup>[9]</sup>. In China, farmers in greenhouse vegetable fields generally adopt excessive fertilizer and frequent irrigation to maintain high yield. Nitrogen is an important nutrient element necessary for plant growth and development<sup>[10]</sup>. However, excessive nitrogen application will cause many negative effects<sup>[11,12]</sup>. Even if it can promote plant growth and increase yield, disease resistance, product quality and water use efficiency of crops may be reduced<sup>[13]</sup>.

Many researchers have studied the effects of excessive application of nitrogen fertilizer on the growth of facility crops<sup>[14,15]</sup>. Zhao et al.<sup>[16]</sup> stated that with the increase of facility agriculture in continuous cropping years, the malondialdehyde (MDA) concentration, superoxide dismutase (SOD) and peroxidase (POD) activity in tomato leaves increased, while the yield and quality decreased. In addition, excessive nitrogen could increase the nitrate content in vegetables, of which the nitrates and nitrites are extremely harmful to human health<sup>[17]</sup>. Soil moisture deficiency

**Received date:** 2020-11-29 **Accepted date:** 2021-07-28

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has a significant impact on plant growth<sup>[18]</sup>. The response to the soil moisture deficiency of plant metabolism is initially manifested as inhibition of cell elongation and growth, resulting in smaller leaves and reduced photosynthetic area. With the increase of soil moisture deficiency, the water potential decreases obviously and the net photosynthetic rate reduces. And the soil moisture deficiency causes plant dehydration, which leads to the destruction of cell membrane structure<sup>[19,20]</sup>. Under normal circumstances, due to the existence of cell membrane structure, different metabolic processes are carried out at different sites and are interconnected. If the membrane structure is disturbed, it will cause the metabolic disorder. Different plant species or cultivars respond differently to soil moisture deficiency. Xerophytes live in the arid environment for a long time and have certain adaptation characteristics in physiology or morphology<sup>[21]</sup>. However, nitrogen and water have a synergistic effect on actual plant production<sup>[22]</sup>. At present, there is still a lack of systematic studies related to the effects of excessive nitrogen fertilizer application and soil moisture deficiency on plant physiological indicators and water relation parameters<sup>[23]</sup>.

There are increasing evidences of the impact of irrigation water quality on crop growth<sup>[24]</sup>. A study applied two water quality treatments (freshwater with EC of 0.86 dS/m, and saline water with EC of 3.6 dS/m) to study the effects of water quality on the yield and quality of tomato plants in Saudi Arabia<sup>[25]</sup>. They reported that the tomato yields and fruit quality decreased due to low-quality water, which can impose a major environmental constraint to crop productivity. The microbiological quality of irrigation water is also very important for it would contribute to the bacterial contamination of fresh vegetables and affect public health<sup>[26]</sup>. Effective microorganisms (EM) technology could relieve plant pathogens and disease, aid the balance and ecology of soil microbes, and improve photosynthetic efficiency and biological nitrogen fixation, etc<sup>[27]</sup>. Therefore, EM irrigation should be beneficial to soil microorganisms, which affects plant physiological and biochemical indicators. However, there are no studies on the effect of EM irrigation on plant growth under the condition of excess nitrogen fertilizer and water deficit. Therefore, the

objective of our study was to investigate the effects of excessive nitrogen fertilizer, soil moisture and microbial diluent on biomass, leaf light response parameters, antioxidant enzyme system activity and osmotic adjustment parameters of tomato seedlings.

## 2 Materials and methods

### 2.1 Experimental site

The experiment was conducted from October to December 2017 in the laboratory of the International Nature Farming Research Center of Japan. The tomato (*Solanum lycopersicum* L. cv. Myoko) was used as plant materials in this experiment.

### 2.2 Irrigation and fertilization treatments

Tomato seedlings (three-true leaf stage) were transplanted into pots with a volume of 280 cm<sup>3</sup> on October 18, 2017. Each pot was filled with 160 g substrate. The basic physical and chemical properties of the substrate were as follows: Organic carbon 4%, pH 6.5±0.7, EC ≤1.5 mS/cm, nitrogen (N)/phosphorus (P)/potassium (K): 150/800/150 mg/L. Each pot was planted with one tomato seedling. There were 80 pots in total. After transplanting, the soil in all the pots was irrigated to 100% of field capacity (FC), and the following days were irrigated according to 70%-80% of FC. After 4 d, tomato seedlings were transferred to the thermostat incubator. Light time was from 6:00 am to 22:00 pm every day, the daytime temperature was 25 °C, and the night temperature was 18 °C. After 11 d, tomato seedlings were treated with different irrigation water, N and microbial diluent. Two irrigation water amounts were set in the experiment, which was respectively 70%-80% (adequate water irrigation) of FC and 50%-80% (water deficiency) of FC. Excessive N application was added 0.585 g pure N per pot (ammonium sulfate and ammonium nitrate were added according to 1:1 content of N). In the control group, except for nutrients in the substrate, no additional nitrogen fertilizer was applied. The irrigation water was treated by using microbial bacteria solution diluted by 300 times (EM bacteria, EM Research Institute of Japan) as the irrigation water, with reference to the control group as tap water. There were eight treatments in total, as listed in Table 1. The treatment finished 50 d after transplanting the tomatoes.

**Table 1 Experiment treatments**

Treatments	Irrigation amount	Nitrogen application rate/g pot <sup>-1</sup>
CK (moderate N)	70%-80% of FC	Nutrients in the substrate
W (water deficiency and moderate N)	50%-80% of FC	Nutrients in the substrate
N (adequate water and excessive N)	70%-80% of FC	0.8357 g NH <sub>4</sub> NO <sub>3</sub> +1.3789g(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , and nutrients in the substrate
WN (water deficiency and excessive N)	50%-80% of FC	0.8357 g NH <sub>4</sub> NO <sub>3</sub> +1.3789g(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , and nutrients in the substrate
EM (EM diluent with adequate water and excessive N)	70%-80% of FC	Nutrients in the substrate
EM-W (EM diluent with water deficiency moderate N)	50%-80% of FC	Nutrients in the substrate
EM-N (EM diluent with adequate water and excessive N)	70%-80% of FC	0.8357 g NH <sub>4</sub> NO <sub>3</sub> +1.3789g(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , and nutrients in the substrate
EM-WN (EM diluent with water deficiency and excessive N)	50%-80% of FC	0.8357 g NH <sub>4</sub> NO <sub>3</sub> +1.3789g(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , and nutrients in the substrate

### 2.3 Plant parameters determination

#### 2.3.1 Leaf photosynthetic light response parameters

The youngest fully expanded leaf was used to determine the photosynthetic parameters by LI-6400 portable photosynthetic instrument (Li. Cor Inc, USA) from 9:00-11:30 am. Each process had five repetitions. Using an open-air path, the light intensity was determined by automatic switching. The chamber temperature was set up to be (22.0±1.5) °C. Photosynthetic photon flux density (PPF) was set as 0, 150, 350, 600, 800, 1200, 1600 and 2000 μmol/m<sup>2</sup> s and the maximum determination time of each light intensity was set as 180 s. The measurement was repeated three times. The analysis model of the light response curve<sup>[28]</sup> is:

$$P_N = P_C (1 - e^{-Ki}) - R_D \quad (1)$$

where,  $P_N$  is the net photosynthetic rate, μmol/m<sup>2</sup> s;  $P_C$  is the maximum net photosynthetic rate, μmol/m<sup>2</sup> s;  $R_D$  is the dark respiration rate, μmol/m<sup>2</sup> s;  $K$  is the half time constant with the maximum quantum yield ( $Y_Q$ ) calculated as  $Y_Q = KP_C$ ; and parameter  $i$  is the photosynthetic photon flux density (PPF), μmol/m<sup>2</sup> s.

#### 2.3.2 Leaf enzyme activity

After 20 d of treatments, 0.1 g of leaf slice was sampled from the youngest fully expanded leaf from the uppermost with repetition three times. The NO<sub>3</sub><sup>-</sup> content of leaf was determined by a reflectometer (RQflex 2, Merck KGaA, Darmstadt, Germany).

At the same time, 0.1 g and 0.5 g of fresh leaf samples were prepared and frozen with liquid nitrogen and stored in a refrigerator at -85 °C. The activity of nitrate reductase (NR) in leaves was determined by sulfonamide colorimetry. The soluble protein was determined by the coomassie bright blue (G-250) method. Superoxide dismutase (SOD) activity was determined by nitroblue tetrazolium test (NBT) colorimetry. Peroxidase (POD) activity was determined by guaiacol oxidation colorimetry. Catalase (CAT) activity was determined by hydrogen peroxide disintegration and iodimetry. Malondialdehyde (MDA) concentration was determined by the trichloroacetic acid method. Superoxide radical was determined by the Bissenbaev method.

2.3.3 Osmotic adjustment analyzed by the pressure-volume curve

After 25 d of treatments, the youngest fully expanded leaf was used to analyze osmotic adjustment by pressure-volume (P-V) curve with a pressure chamber (Model 3000, Soil Moisture Equipment Company, Santa Barbara, California). P-V curve was modeled according to Xu et al<sup>[29]</sup>.

$$-\Psi^{-1} = \{\Psi_{FT}^{-1} - \pi_{s+a}^{-1}[\zeta_0 - \beta(1-\zeta) - \zeta_{ap}]\}e^{-\alpha(1-\zeta)} + \pi_{s+a}^{-1}(\zeta_0 - \beta(1-\zeta) - \zeta_{ap}) \quad (2)$$

where,  $\Psi$  is the leaf water potential at a level of  $\zeta$ ;  $\zeta$  is the relative leaf water content;  $\Psi_{FT}$  is  $\Psi$  at full turgor;  $\pi$  is the osmotic potential;  $\pi_{s+a}$  is  $\pi$  in the symplastic solution theoretically diluted by apoplastic water; parameters  $\alpha$  and  $\beta$  are constants related with the slope at the first part and that at the second part of the P-V curve, respectively, and  $\zeta_{ap}$  is the apoplastic water fraction and it can be expressed by the equation:  $\zeta_{ap} = \zeta_{FT} - \zeta_{sym} = (1 - \zeta_{sym})$ , where,  $\zeta_{sym}$  is the symplastic fraction of  $\zeta$  and  $\zeta_{FT}$  is the  $\zeta$  fraction at full turgor with a value of 1.

The osmotic concentration ( $C_{osm}$ ) was calculated according to Van't Hoff relation<sup>[30]</sup>, Xu et al.<sup>[28]</sup> and Chang et al<sup>[31]</sup>.

2.3.4 Relative water content of leaves

The leaf was sampled and its fresh weight (FW) was weighted immediately. The sampled leaf was hydrated for 24 h at room temperature to weight leaf saturated fresh weight (SW). The leaf dry weight (DW) was tested after being dried by the oven totally.

The relative water content (RWC) was calculated according to the following equation:

$$RWC (\%) = 100 \times (FW - DW) / (SW - DW) \quad (3)$$

2.4 Statistical analysis

Microsoft Excel 2017 was used for data statistical analysis and Origin 2017 was used for drawing the pictures. SPSS 16.0 software was used for one-way analysis of variance. LSD method was used for difference significance tests ( $p < 0.05$ , and  $p < 0.01$ ).

3 Results

3.1 Biomass, relative chlorophyll content (SPAD) and relative water content (RWC) of tomato seedlings

The effects of nitrogen application and irrigation treatments on crop biomass and plant height are shown in Table 2. When the nitrogen application rate was the same, the biomass of each adequate irrigation treatment (i.e., 70%-80% of FC) was higher than that of each soil moisture deficiency treatment (i.e., 50%-80% of FC). When the irrigation amount was the same, the biomass decreased with the addition of N application (such as CK>N, EM>EM-N, W>WN, EM-W>EM-WN). When the amount of irrigation water was the same, the biomass increased with the application of EM (such as EM>CK, EM-N>N). Under the condition of tap water irrigation, compared with CK, the plant dry matter of treatment EM increased by 5.2%, the dry matter of N, and EM-N decreased by 27.0%, and 22.6%, and the plant height decreased by -6.7%, 47.1%, and 37.7%, respectively. It showed that excessive nitrogen application caused plant biomass to decrease, and EM could alleviate this effect. It is probable that microorganisms can take nutrients and elements from the soil in order to improve the growth of crops<sup>[32]</sup>. Under the circumstance of soil moisture deficiency, the plant dry mass of EM-W, WN, and EM-WN decreased by -4.0%, 20.0%, and 28.4% compared to W respectively. The results showed that excessive application of N fertilizer would lead to a decrease in biomass. However, the biomass was not sensitive to microorganisms in this indoor experiment.

**Table 2 Effects of different treatments on crop dry matter weight, plant height, chlorophyll, plant relative water content (RWC), and plant photosynthetic light response parameter**

Treatments	Dry matter/g plant <sup>-1</sup>	Plant/cm	Chlorophyll/OD g <sup>-1</sup>	RWC/%	$P_c/\mu\text{mol m}^{-2} \text{s}^{-1}$	$R_p/\mu\text{mol m}^{-2} \text{s}^{-1}$	$Y_Q/\text{mol mol}^{-1}$
CK	1.15	22.3	34.7	75.2	15.2	2.83	0.032
EM	1.21	23.8	36.9	79.7	16.1	2.69	0.03
N	0.84	11.8	47.6	66.6	9.1	2.27	0.021
EM-N	0.89	13.9	43.8	71.8	9.8	2.14	0.023
W	0.95	18.7	36.7	68.8	12.8	2.21	0.015
EM-W	0.99	18.1	39.2	69.5	13.8	2.72	0.019
WN	0.76	11.7	48.9	69.5	4.9	1.87	0.014
EM-WN	0.68	12.5	44.6	72.3	5.8	1.93	0.018
Irrigation amount	*	**	**	**	**	**	**
Nitrogen application	ns	ns	ns	**	*	*	**
Irrigation x Nitrogen	ns	ns	ns	*	ns	ns	ns

Note: \* and \*\* indicate that the correlation is significantly different at the levels of  $p=0.05$  and  $0.01$ , respectively; ns indicates that the difference is not significant. The same as below.

Chlorophyll is the material basis of photosynthesis reflecting the physiological status of photosynthetic system. When the irrigation amount was the same, chlorophyll values of N and WN were respectively higher than the control group, CK and W (Table 2). It indicated that under the circumstance of adequate 1 irrigation amount or soil moisture deficiency conditions, excessive nitrogen application could both increase the amount of chlorophyll. The chlorophyll values with adequate irrigation amount from high to low were N, EM-N, EM and CK, and with water deficiency from

high to low were WN, EM-WN, EM-W and W. The results showed that single application of EM could improve the SPAD values of plants to a certain degree, but the combination of microbial diluent and excessive nitrogen fertilizer were applied together may lead to the decrease of chlorophyll.

Statistical analysis showed that the effects of irrigation amount on dry matter reached a significant level ( $p < 0.05$ ), and the influence on plant height, chlorophyll and relative water content reached an extremely significant level ( $p < 0.01$ ). The influence of

nitrogen application on relative water content reached an extremely significant level ( $p < 0.01$ ), and the influence on chlorophyll did not reach a significant level. The interaction between irrigation amount and nitrogen application on RWC reached a significant level ( $p < 0.05$ ), while the interaction between other indexes was not significant ( $p > 0.05$ ). The influence of EM on various indicators and its interactions with nitrogen fertilizer and water deficit were neglectable, therefore, the significance analysis was not shown here.

### 3.2 Light response parameters

As shown in Table 2, excessive nitrogen application decreased the leaf photosynthetic capacity ( $P_c$ ), such as  $P_c$  of N, EM-N, WN and EM-WN were 40.1%, 35.5%, 67.8% and 61.8% lower than that of CK, EM, W and EM-W, respectively. Compared to CK, N, W and WN, the  $P_c$  of EM, EM-N, EM-W and EM-WN increased by 5.9%, 7.7%, 7.8%, and 18.4%, respectively. In consideration of the factor of different amounts of irrigation water, the  $P_c$  of the four treatments with adequate amount (CK, EM, N, EM-N) were higher than that of the four treatments with water deficiency (W, EM-W, WN and EM-N), and the former four treatments (adequate water) improved 18.8, % 16.7%, 85.7% and 69.0% respectively. The results showed that the application of nitrogen, EM, and irrigation amount affects  $P_c$ . In addition, the  $P_c$  values in EM and EM-W plots were higher than in other treatments under adequate water irrigation conditions.  $P_c$  reached the maximum value ( $16.1 \mu\text{mol}/\text{m}^2 \text{ s}$ ) under the condition of adequate irrigation plus EM application. It indicated that the combination of adequate water irrigation and EM benefited the leaf photosynthetic potential in this experiment.

The dark respiration rate ( $R_D$ ) of the N treatment increased by 21.39% more than that of the WN, treatment and the  $R_D$  of EM-N treatment increased by 10.88% more than that of the EM-WN treatment (Table 2). The results showed that the single water stress and combination of EM and water stress could both lead to

the increase of leaf  $R_D$  under the condition of excessive nitrogen application. Under the condition of excessive application of N, the dark respiration rate (RD) of N was larger than WN, and  $R_D$  of EM-N was larger than EM-WN (Table 2). With adequate irrigation, except CK, the  $R_D$  of EM was the highest ( $2.69 \mu\text{mol}/\text{m}^2 \text{ s}$ ). Under the condition of water deficiency, the EM-W had the highest RD value ( $2.72 \mu\text{mol}/\text{m}^2 \text{ s}$ ) among the four treatments. The results showed that excessive nitrogen application, water deficit and EM application had different effects on leaf light response parameters.

Statistical analysis showed that the effects of irrigation amount on photosynthetic activity reached an extremely significant level ( $p < 0.01$ ). The influence of nitrogen application on  $Y_Q$  reached an extremely significant level ( $p < 0.01$ ), the influence on  $P_c$  and  $R_D$  reached a significant level ( $p < 0.05$ ). The interaction between nitrogen application and irrigation amount was not significant ( $p > 0.05$ ). The interactions of EM with other treatments were not analyzed here.

### 3.3 Nitrate reductase activity

As shown in Table 3, there are differences in the nitrate reductase (NR) activities among different treatments. Under different nitrogen application conditions, the NR of treatment WN had the highest value, which increased by 34.0% compared with W, the NR of treatment N increased by 13.0% compared with CK, the NR of EM-N increased by 22.9% compared with EM, and the NR of EM-WN increased by 28.6% compared with the EM-W. It indicated that the NR activity of excessive N application was higher than the non-N treatments. The amount of irrigation water had an impact on NR activity, such as the NR of W was lower than CK, and the NR values of EM-W, WN, EM-WN were higher than EM, N and EM-N, respectively. With the application of EM, the NR of EM, EM-N, EM-W and EM-WN decreased by 11.1%, 3.3%, 7.5% and 11.3% compared with CK, N, W and WN, respectively. The results showed that EM did not enhance the activity of NR.

**Table 3 Effects of different treatments on plant nitrate reductase (NR),  $\text{O}_2^-$ , and enzyme activity (MDA, SOD and POD)**

Treatments	NR/ $\mu\text{mol h}^{-1} \text{ g}^{-1}$	$\text{O}_2^-/\text{nmol g}^{-1}$	MDA/ $\mu\text{mol g}^{-1}$	SOD/OD $\text{g}^{-1}$	POD/OD $\text{min}^{-1} \text{ g}^{-1}$	CAT/OD $\text{min}^{-1} \text{ g}^{-1}$
CK	0.54	149.7	0.017	0.042	80.4	147.8
EM	0.48	92.6	0.012	0.062	182.8	226.2
N	0.61	183.7	0.01	0.075	162.8	150.2
EM-N	0.59	130.3	0.012	0.078	146.7	233.9
W	0.53	202	0.015	0.042	71.8	133.8
EM-W	0.49	98.3	0.014	0.048	69.8	204.6
WN	0.71	164.2	0.013	0.087	258	221.4
EM-WN	0.63	126.6	0.012	0.075	232.4	142.1
Irrigation amount	**	*	**	**	**	**
Nitrogen application	**	ns	ns	ns	*	*
Irrigation $\times$ Nitrogen	ns	ns	ns	ns	ns	ns

### 3.4 Active oxygen, concentration of MDA and activities of antioxidant enzymes

The leaf  $\text{O}_2^-$  of treatment W was 34.9% higher than that of CK, but MDA was 11.8% lower than that of CK, which showed that soil moisture deficiency increased the synthesis of  $\text{O}_2^-$  causing the damage to plants, but decreased the synthesis of MDA to relief this damage (Table 3). The  $\text{O}_2^-$  of EM application treatments (i.e., EM, EM-N, EM-W and EM-WN) were higher than that of corresponding treatment without application treatments (i.e. CK, N, W and WN), but generally, the MDA of the former treatments (except EM-N) were lower than that of the latter treatments.

When excessive nitrogen was single applied (no EM and adequate water), the  $\text{O}_2^-$  content of treatment N increased 22.7% compared to CK, and the MDA of N was 4.11% lower compared to

CK. And the  $\text{O}_2^-$  and MDA contents of treatment W were higher than that of WN under soil moisture deficiency conditions. It indicated that under the condition of adequate irrigation amount, the application of excessive nitrogen increased the synthesis of intracellular  $\text{O}_2^-$  apparently, and decreased MDA a little, which increased the damage to plants generally. However, when water stress was superimposed, the synthesis of  $\text{O}_2^-$  and MDA decreased, which could be relieved the damage caused to plants. Compared with CK, the  $\text{O}_2^-$  and MDA of treatment EM-N were reduced by 13.0% and 29.4%, respectively; compared with treatment W, the  $\text{O}_2^-$  and MDA of EM-WN were reduced by 37.3% and 20.0%, respectively. However, the  $\text{O}_2^-$  of EM-N was higher than that of EM and had the same MDA; in addition, the  $\text{O}_2^-$  of EM-WN was higher than that of EM-W, and MDA was slightly lower. This

showed that, compared with excessive application of nitrogen alone, the combined application of EM and N increased leave  $O_2^-$ , and EM and excessive nitrogen application had no synergistic effect on the elimination of  $O_2^-$  and MDA. Among all the treatments, EM treatment had the lower contents of  $O_2^-$  and MDA, such as EM<CK. And the  $O_2^-$  and MDA contents were not sensitive to the irrigation water conditions. EM could most effectively prevent increases in leaf membrane lipid peroxidation and enhance the adaptability to the drought environment in tomato seedlings compared to soil moisture deficiency and excessive nitrogen application.

As shown in Table 3, the irrigation amount has different effects on the antioxidant enzyme activities (SOD, POD and CAT) in tomato seedlings. Compared with CK, the SOD activity of treatment W was equal to it, while the POD activity and CAT activity decreased by 10.7% and 9.5%, respectively. The SOD, POD and CAT of treatment EM-W were 22.6%, 61.8% and 9.6% lower than treatment EM, respectively. Application of EM increased the activity of SOD, POD and CAT (such as treatment EM>CK). In addition, EM also increased the activity of SOD and CAT (such as EM-N>N, EM-W>W) under conditions of single nitrogen stress and single water deficit. This showed that EM had a certain effect on enhancing the activity of antioxidant enzymes. When tomato seedlings produced more  $O_2^-$  and  $H_2O_2$ , EM could stimulate the tomato's self-protection mechanism, induce enhanced protective enzyme activity, and improve crop resistance. However, activities of SOD, POD and CAT in treatment EM-WN were lower than that of WN, indicating that under the combined stress of water deficit and excessive nitrogen fertilizer, EM had a minor effect on the improvement in antioxidant enzyme activities. In general, the application of excessive nitrogen fertilizer increased the leaf antioxidant enzyme activities. For example, compared with CK, activities of SOD, POD and CAT of treatment N increased by 78.5%, 102.5% and 1.6%, respectively. Compared with EM-W, activities of SOD and POD of EM-WN increased by 56.3% and 232.3%, respectively, concluding that under the conditions of water deficit and EM application, the application of excessive nitrogen fertilizer can obviously increase the antioxidant enzyme activities.

Statistical analysis showed that the influence of irrigation amount on NR, MDA, SOD, POD and CAT reached an extremely significant level ( $p<0.01$ ), and the influence on  $O_2^-$  reached a significant level ( $p<0.05$ ). The influence of nitrogen application on NR reached an extremely significant level ( $p<0.01$ ), the influence on POD and CAT reached a significant level ( $p<0.05$ ), and the influence on  $O_2^-$ , MDA and SOD did not reach a significant level. The interaction between irrigation amount and nitrogen

application soil on NR,  $O_2^-$ , and enzyme activity was not significant ( $p>0.05$ ). The interactions of EM with other treatments were not analyzed here.

### 3.5 Osmotic adjustment induced by soil moisture deficiency and excessive nitrogen application

#### 3.5.1 Changes in osmotic potential $\pi_{FT}$ and $\pi_{IP}$

As shown in Table 4, regardless of adequate irrigation or under water deficit conditions, excessive nitrogen application resulted in a decrease in the  $\pi_{FT}$  concentration, while an increase in the cytosolic solute concentration ( $C_{osm}$ ) and concentration difference ( $\Delta C_{osm}$ ). This showed that the application of excessive nitrogen led to the accumulation of soluble substances, and the increase in cell fluid concentration enhanced its ability to maintain the maximum turgor pressure to resist a certain degree of drought stress from the outside environment. Under the same nitrogen application level, water deficit reduced  $\pi_{FT}$  by increasing  $C_{osm}$  and  $\Delta C_{osm}$ . For example, compared with N, the  $\pi_{FT}$  in WN reduced by 11.3%, and  $C_{osm}$  and  $\Delta C_{osm}$  increased by 5.27% and 27.5%, respectively.

The  $\pi_{IP}$  value reflects the plant's ability to maintain turgor pressure and the plant's drought tolerance. From Table 4, it can be seen that with the same irrigation level, the  $\pi_{IP}$  values were as follows: CK>N and W>WN, indicating that excessive nitrogen application improved the osmotic adjustment ability of tomato seedlings to varying degrees. Under the same nitrogen application, the  $\pi_{IP}$  of treatment W was lower than CK, and the  $\pi_{IP}$  of treatment WN was also lower than N. Significance analysis results showed that excessive nitrogen application ( $p<0.05$ ) and water deficit ( $p<0.01$ ) significantly affected the ability of tomato seedlings to maintain maximum turgor pressure, but the synergy between the two was not significant.

The greater the difference between the two values of  $\pi_{FT}$  and  $\pi_{IP}$ , the stronger the osmotic adjustment ability of the plant. Compared with moderate nitrogen application treatments (CK and W), the difference of excessive nitrogen treatments (N and WN) increased by 5.89% on average; compared with traditional irrigation (CK and N), the difference of water deficit treatments (W and WN) increased by 67.9% on average. It showed that excessive nitrogen application and water deficit can induce the osmotic adjustment ability to be improved to different degrees.

Statistical analysis showed that the influence of irrigation amount on osmotic adjustment parameters reached an extremely significant level ( $p<0.01$ ), and the influence of nitrogen application reached a significant difference ( $p<0.05$ ), but the interaction between the two was not significant ( $p>0.05$ ). The interaction of EM with other treatments was not analyzed here.

**Table 4 Parameters of osmotic adjustment of tomato seedlings under different irrigation and nitrogen fertilizer application conditions (P-V curve method)**

Treatments	Irrigation amount	Nitrogen application	$\Psi_{FT}$	$\pi_{FT}$	$P_{FT}$	$\pi_{s+a}$	$\pi_{IP}$	$\zeta_{IP}$	$\zeta_{sym}$	$C_{osm}$	$\Delta C_{osm}$
CK	70%-80% of FC	No	-0.208	-0.831	0.622	-0.588	-1.108	0.823	0.698	340.7	
N		Yes	-0.222	-0.872	0.651	-0.633	-1.169	0.859	0.778	357.5	16.8
W	50%-80% of FC	No	-0.238	-1.052	0.814	-0.756	-1.522	0.775	0.721	431.3	90.6
WN		Yes	-0.247	-1.112	0.865	-0.795	-1.606	0.788	0.819	455.9	115.2
	Irrigation amount		*	**	**	**	**	**	**	**	**
	Nitrogen application		ns	ns	ns	*	*	*	**	ns	ns
	Irrigation × Nitrogen		ns	ns	ns	ns	ns	*	ns	ns	ns

Note:  $\Psi_{FT}$ ,  $\pi_{FT}$  and  $P_{FT}$  are the leaf water potential, osmotic potential and turgor pressure during water saturation respectively;  $\pi_{s+a}$  is the osmotic potential of the plastid diluted by the apoplast;  $\pi_{IP}$  is the osmotic potential during plasmolysis;  $\zeta_{IP}$  is the relative water content during plasmolysis;  $\zeta_{sym}$  is the relative water content of the symplast;  $C_{osm}$  is the osmotic concentration and  $\Delta C_{osm}$  is the active increase in  $C_{osm}$  due to the osmotic adjustment.

3.5.2 Changes in the relative water content at plasmolysis ( $\zeta_{IP}$ ), symbiotic water fraction ( $\zeta_{sym}$ ) and apoplast water fraction ( $\zeta_{ap}$ )

The parameter  $\zeta_{IP}$  is the leaf relative water content at plasmolysis. In other words, when the plant is dehydrated and leaf relative water content decreases to the point of  $\zeta_{IP}$ , the leaf lose its turgor completely and the cell walls separate from the membrane of cytoplasm (or symplasm). Therefore, the lower  $\zeta_{IP}$  is, the higher the tolerance of the plant to water stress.  $\zeta_{sym}$  or  $\zeta_{apo}$  is the fraction or proportion of the water content in cell symplasm or apoplast against the total cell water. Usually, the higher  $\zeta_{sym}$  is, the higher the tolerance of tissue cells to dehydration<sup>[33]</sup>. Table 4 shows that under the same nitrogen application conditions, water deficit treatments reduced leaf  $\zeta_{IP}$  and increased  $\zeta_{sym}$ . Such as, the  $\zeta_{IP}$  of treatment W and WN were lower than that of CK and N, and  $\zeta_{sym}$  were higher. The results showed that water deficit imposed an acclimation effect on the plants and increased the tolerance of leaves to dehydration. In addition, it is believed that the higher the  $\zeta_{sym}$  ( $\zeta_{sym}=1-\zeta_{apo}$ ), the stronger the viscous cell protoplast and the stronger the hydrophilicity of the protoplast colloid, which is conducive to water absorption and water retention by plants. Both water deficit and excessive nitrogen application increased  $\zeta_{sym}$  and decreased  $\zeta_{ap}$ , indicating that both water deficit and excessive nitrogen application increased the water absorption and water retention capacity of tomato leave. However, excessive nitrogen application increased  $\zeta_{IP}$  and it was speculated that excessive nitrogen could decrease  $\zeta_{sym}$ , for example,  $\zeta_{IP}$  increased by 0.036 MPa and  $\zeta_{sym}$  increased by 0.08. excessive nitrogen application resulted in a decrease in tolerance of leaves to dehydration because it increased  $\zeta_{IP}$  under the same irrigation treatment.

## 4 Discussions

### 4.1 Photosynthetic response parameter

The maximum net photosynthetic rate ( $P_C$ ) is an important indicator for determining the photosynthetic potential of leaves. In this experiment,  $P_C$  values of EM and EM-W are higher than those of other treatments. It is probably because EM improves the capacity of photosynthesis and enhances the adaptability of tomato plants to drought environment<sup>[34-37]</sup>. Under the condition of excessive application of N,  $P_C$  values of N and WN are lower than other treatments. Presumably, nitrogen fertilizer could change the adaptation range of tomato to light and decrease the adaptability to the light environment. Therefore, excessive nitrogen fertilizer is not conducive to improving the light use efficiency of plants<sup>[38,39]</sup>. When the excessive nitrogen and EM are applied together,  $P_C$  of EM-N and EM-WN are slightly higher than that of N and WN. But, much lower than that of CK and W. It indicates that there is no synergistic effect between excessive nitrogen fertilizer and microorganisms. The combined application of them would decrease the light use efficiency of plants.

Under the condition of excessive nitrogen and EM application, the  $R_D$  value of adequate irrigation amount (EM-N) is higher than that of soil moisture deficiency (EM-WN). This may be because tomatoes grow better under adequate water irrigation, therefore, the rate of photosynthate consumption was faster<sup>[40]</sup>. In the adequate irrigation amount conditions, the  $R_D$  value of CK is the largest, while under soil moisture deficiency conditions, the  $R_D$  value of EM-W is the largest. It is probably that the increase of the dark respiration rate of the microbial treatment stimulates regulation of stomatal opening of the crop. And it will reduce the excessive transpiration<sup>[41]</sup>, which does not significantly affect the net photosynthetic rate ( $P_N$ ). However, under the condition of

excessive application of nitrogen, the effect of EM on plant  $R_D$  is not irregular, may increase or decrease. Therefore, the effect of microorganisms on plant  $R_D$  needs further study, such as the setting of different microbial dosages.

### 4.2 Antioxidant enzyme system and nitrate reductase (NR)

In our experiment, excessive nitrogen application increases NR activity. It may be due to excessive nitrogen fertilizer, which accelerates the synthesis of nitrate reductase in plants to cope with adversity stress<sup>[42,43]</sup>. When nitrogen and EM application are the same, soil moisture deficiency increases the activity of NR enzymes. The reason may be the same as above. Under different microorganisms' conditions, the NR activity of the plants treated with EM slightly decrease. This may be because EM does not cause stress on plant growth, so it has no obvious effect on NR activity.

Soil moisture deficiency and excessive nitrogen application results in a sharp increase in  $O_2^-$  and MDA content in plant leaves. It is probably due to the roots are subjected to soil moisture deficiency, which results in increasing the production rate of  $O_2^-$  and the degree of membrane lipid peroxidation. Whether single soil moisture deficiency, single excessive nitrogen or combined stress of them, the application of EM decreases the  $O_2^-$  and MDA content of plants. It can be observed that EM can decrease the degree of membrane lipid peroxidation and enhance the adaptability of tomato seedlings to the arid environment.

In the long-term evolution process, plant cells form an antioxidant enzyme system that protects against active oxygen ion toxicity. Superoxide dismutase (SOD) and peroxidase (POD) are important antioxidant enzymes in the antioxidant system of plants, which are to eliminate active oxygen ( $O_2^-$ ). Catalase (CAT) and peroxidase (POD) are ubiquitous in animal and plant cells, which are mainly to promote the degradation of  $H_2O_2$  in order to decrease the damage of  $H_2O_2$  to the biofilm system. In this experiment, the activities of SOD, CAT, POD and NR increased obviously in the leaves of tomato seedlings with excessive application of nitrogen. It indicates that the increasing of antioxidant enzyme activities slows down or resists the adversity stress on plant growth caused by excessive application of N<sup>[44]</sup>.

### 4.3 P-V curve water parameters

In this study, whenever under adequate irrigation or water deficiency,  $\pi_{FT}$  of the treatments with excessive nitrogen fertilizer (WN and N) are all lower than that of the treatments without N fertilizer (W and CK). In addition, the concentration of cytoplasmic solute ( $C_{osm}$ ) and concentration difference ( $\Delta C_{osm}$ ) increases apparently. At the same time, the soil moisture deficiency follows the same change law. It may be because the application of excessive nitrogen leads to the accumulation of soluble substances and the increase of cell concentration, thus enhancing the ability to maintain maximum turgor pressure in order to counteract a certain degree of drought stress.

According to the previous analysis, the same treatment has different effects on various plant osmotic adjustment parameters. Therefore, it's necessary so to comprehensively evaluate the ability of plants to maintain turgor pressure. The subordinate function values of different irrigation and nitrogen application treatments are calculated and shown in Table 5. The average function value of WN is the highest (0.8557), followed by W (0.7430) and N (0.2484), and the smallest is CK (0.1786). It shows that the WN has the strongest comprehensive ability to maintain turbulence, and CK has the worst adaptability to bad environment. This means

that under water deficit conditions, applying excessive nitrogen treatment can effectively enhance the ability of tomato seedlings to maintain turgor pressure and improve their adaptability under adverse environments. It is inferred that deficit irrigation on the soil where excessive fertilization occurs may increase the absorption of nitrogen by plants, resulting in excessive nitrate in fruits and leaves. In practice production, reasonable deficit irrigation systems need to develop according to different soil salinization degrees and soil and crop types.

**Table 5 Subordinate function values calculated by osmotic adjustment parameters**

Parameter	Function values			
	CK	N	W	WN
$\psi_{FT}$	0	0.3590	0.7692	1
$\pi_{FT}$	0	0.1459	0.7865	1
$P_{FT}$	0	0.1193	0.7901	1
$\pi_{s+a}$	0	0.2174	0.8116	1
$\zeta_{IP}$	0.4286	0	1	0.8452
$\zeta_{sym}$	1	0.3388	0.8099	0
$C_{osm}$	0	0.1458	0.7865	1
$\zeta_{ap}$	0	0.6612	0.1901	1
Mean value	0.1786	0.2484	0.7430	0.8557

## 5 Conclusions

In this study, the effects of excessive nitrogen fertilizer, soil moisture deficiency and microbial diluent on tomato seedlings growth, leaf light response parameters, antioxidant enzyme system activity and osmotic adjustment parameters were studied in indoor experiments. The results showed that the biomass (dry weight and plant height) was reduced with single excessive nitrogen, single soil moisture deficiency, or a combination of them. Excessive nitrogen can increase the relative content of chlorophyll (SPAD) apparently, while soil moisture deficiency improves the chlorophyll minorly. Both soil moisture and nitrogen fertilizer significantly affect photosynthetic characteristics, which is manifested in the decrease of PC, RD, and YQ, but the application of EM can alleviate it to a certain extent. Application of excessive nitrogen and EM could increase the activity of antioxidant enzymes (SOD, POD and CAT) and NR in tomato seedling leaves. And antioxidant enzymes and NR were significantly affected by soil moisture deficiency. Soil moisture and excessive nitrogen fertilizer significantly affect plant osmotic adjustment (such as  $\pi_{FT}$ ,  $C_{osm}$ ,  $\Delta C_{osm}$  and  $\zeta_{ap}$ ), and reduced the water absorption and water retention capacity of tomatoes to a certain extent.

## Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (51509068) and Fundamental Research Funds for the Central Universities (B200202093).

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