Osmotic adjustment and up-regulation expression of stress-responsive genes in tomato induced by soil salinity resulted from nitrate fertilization

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Abstract: Concerns about the soil salinity caused by excessive fertilization have prompted scientists to clarify the detailed mechanisms and find techniques to alleviate the damage caused by this kind of soil salinity. Aims of this study were to elucidate the effect of soil salinity caused by nitrate fertilization and the differences in salinity effect between nitrate salts and NaCl salt with analyses at various levels of crop physiology and molecular biology. A microbial inoculation was also tried to verify whether it could alleviate the salinity-induced loss and damages. In three experiments (Exp I, II and III), nitrate salts (NS) of $Ca(NO_3)_2$ and KNO_3 were applied to potted tomato plants to simulate the soil salinity caused by fertilization and a microbial inoculant (MI) was applied. Photosynthesis was measured using Li-6400. Osmotic adjustment was analyzed using the mathematically modeled pressure-volume curve; O_2^- concentration and activity of SOD and nitrate reductase were measured. Expression of nitrate reductase gene and the stress-responsive gene *DREB2* was analyzed using the real-time PCR method. In Exp I and II, where the applied NS amount was moderate, NS application at low concentration induced increases in O_2^- and MDA concentrations and plants acclimated to the soil salinity as the treatment prolonged for weeks. The acclimation was contributed by osmotic adjustment, activation of SOD and re-compartmentation of cell water between symplasm and apoplasm. These adjustments might be ultimately attributed to up-regulation of stress-responsive genes such as *DREB2* as well as the nitrate reductase (NR) gene. However, in Exp III, applications of NaCl and NS at high concentration could not show positive effects as NS did. Application of MI synergistically increased the xerophytophysiological regulation caused by NS and alleviated the salinity damage in addition to its own positive effects on the tomato plants. Different from NaCl, nitrate salts at low application rate increased the total biomass and fruit yield of tomato and induced up-regulation expression of stress-responsive genes and the consequent active osmotic adjustment. However, nitrate application at high level negatively affected tomato plants irrespective of the gene up-regulations. Application of MI alleviated the salinity damage and synergistically increased the xerophytophysiological regulation caused by the soil salinity in addition to its positive effects on the tomato crop but the detailed mechanisms needed to be clarified in future further studies.

Keywords: nitrate fertilizer, osmotic adjustment, salinity stress, soil salinization, tomato (Solanum lycopersicum); xerophytophysiology, microorganism, bioremediation

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1 Introduction

Nitrate is the primary form of nitrogen nutrient in agricultural soils[1]. However, one of the consequences of the excessive application of nitrogen fertilizers is soil salinization caused by nitrate accumulation[2-4] in addition to food contamination[5] and environment pollution[6,7]. Production practices and studies by pioneers have found these problems and these problems have prompted scientists and policymakers to conduct field surveys and experiments to examine the impact of nitrate accumulation on environment and food quality, especially the secondary soil salinization. Now it is clearly known that the excessive nitrate accumulation is mainly caused by input of nitrogen fertilizers and animal manures at rates higher than crop requirements. Unfortunately, this problem has not yet been payed enough attention. Actually, the effect of fertilizers on soil salinity is not immediately obvious because the soil has strong buffering capacity to its salt content[8,9]. However, long-term overload of chemical fertilization has caused soil degradation and loss of its buffering capacity. Soil salinization is often attributed to sodium and potassium contained in fertilizers. However, it is neglected that

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nitrate must be paired with cations at existence in soils. Obviously, excessive nitrate accumulation contributes to soil salinization no matter whether cations or nitrate impose more effect on the salinity[10]. Accumulation to high level of nitrate in soils may be caused by excessive applications of nitrogen fertilizers, in either urine or nitrate form, and even in organic forms. Nitrate at high concentration causes soil salinity shown by high soil EC and many other problems in crop production, especially in soil-based greenhouses[12-14]. In the extreme case in China, 15 tons of chemical fertilizers and 30 m^3 of compost are applied to 1 ha of soil-based greenhouse land to ensure the highest yield of vegetable through the whole year[13,14]. Therefore, farmers have to totally change the greenhouse soils every three years to solve the salinity problems[15]. Different from cations such as sodium that cause soil salinity, nitrate is the essential nutrient to plants in a hand although its excessive accumulations in soils and food plants cause profound problems in environment and in food quality[7]. Soil salinity causes plant water stress, ionic imbalance, mineral nutrition deficiency, photosynthetic inhibition, carbon allocation and utilization and the final reduction in productivity of crops including tomatoes[16-19]. Nevertheless, as one of the soil salinity source, nitrate is different from the cations such as Na^+ and K^+[20] because nitrate is a main essential nutrient to plants[10]. It is difficult to separate the injurious effect caused by salinity from the positive nutritional effect of nitrate. It is important to clarify how nitrogen fertilization causes soil salinization and what consequence in plant growth and physiology would be induced by the soil salinity caused by the excessive nitrate accumulation. Therefore, in the present study, the focus was placed on the soil salinity caused by excessive nitrate application and its effect on plant growth and physiology in potted tomato crops, in aspects of photosynthetic activities, turgor maintenance from osmotic adjustment, and the final fruit yield. Moreover, excessive accumulation of nitrate in soil may also cause excessive accumulation of nitrate in plant body, which may interfere with nitrogen metabolism. The nitrogen metabolism started from nitrate and nitrate reductase (NR) occupies a central position in nitrogen metabolism in higher plants[21,22]. The nitrate reductase is encoded by the NR gene[23,24]. Although the nitrate reductase and the NR gene are subjected to complex regulation mechanisms, the activity of the enzyme and expression of the gene are induced and regulated by nitrate itself[25-29]. Therefore, in the present study, activity of nitrate reductase and the expression of the gene coding for NR were examined to elucidate the effect of nitrate accumulation in soil. Moreover, as mentioned above, salinity caused by excessive nitrogen fertilization may induce xerophytophysiological regulations such as osmotic adjustment and the consequent leaf turgor maintenance in order to alleviate damages by the stresses. These adaptation and adjustment processes are ultimately controlled by the stress responsive genes, one of which is *DREB2*[30]. In the first step, crisis signal substances including abscisic acid, jasmonic acid and oxygen radical are induced and a cascade of signal transduction is involved[31]. One of the easily detected signal substances is O_2^-. Accumulation of O_2^- may induce synthesis and activation of the antioxidant enzymes such as SOD and in turn the antioxidant enzymes can delete O_2^- and damages are avoided or alleviated[32]. Therefore, in the present study, antioxidant function and induced up-regulation express of the stress-responsive gene were examined. Many practice have been tried to alleviate the injurious effect of soil salinity, by providing adequate drainage[33], growing salt tolerant varieties[34], applying inorganic and organic amendments, and maintaining adequate soil moisture[35]. There are also many research cases using microbial materials in alleviating soil salinity. However, the microbes used are different in their sources and effectiveness. Bashan[36] evaluated bacterial inoculants used in agriculture with emphasis on semiarid agriculture including salinity and drought stresses. Dodd and Perez-Alfocea[37] indicated the beneficial physiological effects of microorganisms including improved nutrient and water uptake, growth promotion, and alteration of plant hormonal status and metabolism by alleviating stresses such salinity, with special reference to signaling mechanisms that interact with key physiological processes to improve plant tolerance to the osmotic and toxic components of salinity. They conclude that the improved plant nutrition is a general beneficial effect and may contribute to the maintenance of homeostasis of toxic ions under saline stress. Mayak[38] reported that bacteria populating arid and salty environments conferred resistance in tomato and pepper plants to water stress. They found one of these strains, *Achromobacter piechaudii* ARV8, which significantly increased the fresh and dry weights of both tomato and pepper seedlings exposed to transient water stress. In the present study, a microbial inoculant, with yeast, lactic acid bacteria and actinomycetes as the main species, was tried as an amendment to alleviate the soil salinity and the injurious effects caused by excessive nitrate accumulation in soil. This inoculant has been used by farmers in Japan and many other countries although the related research lags behind. Xu's research group had tried this microbial inoculant in saline soil improvement in the Yellow River Delta area and found that it was effective in alleviation of soil salinity by improving soil aggregation[39]. However, it is not clear whether the same microbial inoculant is effective in alleviation of the secondary salinity caused by excessive use of nitrogen fertilizers. Therefore, in the present study, the microbial inoculant was just first used as the practice to test whether it could reduce the salinity injury and the detailed mechanisms may be considered in the future research.

2 Materials and methods

2.1 Plant materials and treatments

Three experiments (Exp I, II and II) were conducted with potted plants of tomato (*Solanum lycopersicum* L. cv. Myoko) with treatments changed in different experiments.

2.1.1 Exp I

Tomato seedlings were transplanted in Wagner's pots with a soil surface of 0.05 m^2 and a height of 30 cm in early July and grown under a rainout shelter. The soil is an Andosol and the chemical properties before fertilization, after treatment and after plant harvest are presented in Table 1. The soil in each pot was fertilized with 45 g of organic fertilizer (N, 5.2, P, 30, K, 20 g/kg), which was fermented with oil mill sludge, rice bran and fish meal. Because the treatments were related with chemical fertilizer application, in plots of the control (CK) chemical fertilizers were avoided and the organic fertilizer was applied to the soil as the base fertilization to support the basic plants growth. Four treatments as 2 (salinity) ×2 (microorganisms) factorial were designed as follows: 1) Salinity: salt content of 3.0 g/kg by applying 4.2 g $Ca(NO_3)_2$ and 4.2 g KNO_3 to each pot; 2) Salinity-MI: Treatment 1) with 10 ml of microbial inoculant (MI) applied into the soil; 3) CK: without salt application; 4) CK-MI: CK with 10 ml of MI applied. The microbial inoculant contains lactic bacteria (*Lactobacillus plantarum*), yeasts (*Saccharomyces cerevisiae*), actinomycetes (*Streptomyces albus*) and photosynthetic bacteria

(*Rhodopseudomonas palustris*) as main components at pH 3.5 with a commercial name as EM (EM Laboratory Co., Ltd., Shizuoka, Japan). The four treatments were arranged in a 4×4 Latin Square under a rainout shelter with 8 pots per plot. The microbial inoculant (MI) is developed by International Nature Farming Research Center (Matsumoto city, Nagano, Japan) containing a group of beneficial microorganisms as lactic acid bacteria and yeast[40]. The liquid product of this microbial inoculant reaches pH 3.5 with total microbial density within 10^{10}-10^{12} CPU mL^{-1}. Five weeks after seedlings transplanted, the plants entered the flowering stage. Then, treatments were imposed to the pots. Samples were taken from the 5th leaf from the top 6 h, 7 day and 35 days after treatments started. The sample used for nitrate reductase activity measurement was 0.5 g and that for RNA extraction was 0.1 g. Leaf photosynthesis was measured 30 days after treatments started and analyses of Pressure-Volume curve and excised leaf transpiration declining curve were also conducted at this time.

2.1.2 Exp II

Experiment was conducted in artificial growth chambers inside laboratory to confirm Exp I only at levels of the molecular biology and biochemistry with young plants. Other factors such as fruit yield and soil properties were not considered in this small pot experiment. The light intensity was 160 μmol/m^2·s over the canopy of the tomato seedlings. Temperature was controlled at (22±1/20±1)°C (day/night). The relative air humidity was (60±1)%. Polyethylene pots with a soil surface of 0.001 m^2 and a height of 15 cm. The pots were filled with a commercial peat-moss based substrate with N, P and K (N-P-K=150-800-150 mg/L) fertilized ready. In addition, treatments were made as follows:1) CK: without additional fertilization, 2) NS: the soil salt concentration of 30 g/kg with $Ca(NO_3)_2$ and KNO_3, 3) CS: the soil salt concentration of 30 g/kg with $CaCl_2$ and NaCl, 4) MI, 5) MN and 6) MC were added with microbial inoculant (MI, the same as in Exp I) in addition to 1) CK, 2) NS and 3) CS. The microbial inoculate in ml was diluted 100 times added to each pot of treatments of 4), 5) and 6). Leaf sample used for analyses in biochemistry and molecular biology were taken 3 d, 8 d and 15 d after treatments started. Photosynthesis measurement and analyses of Pressure-Volume curve and excised leaf transpiration declining curve were made 21 d after treatments started.

2.1.3 Exp III

Small plastic pots were used as the same in Exp I. The purpose of Exp III was used to firm Exp II at a further higher salinity level. Management of plant materials and examined variables were the same as described in Exp II with only concentration of salts doubled.

2.2 Measurement of leaf photosynthesis and leaf color

Leaf photosynthesis was measured and analyzed according to Xu et al.[40]. Leaf color was measured in situ using a chlorophyll meter (SPAD-502, Konica Minolta, Osaka, Japan).

2.2.1 Measurement of glucose concentrations in fruit

Glucose were measured by a reflectometer (RQflex 10, Merck KGaA, Darmstadt, Germany) following the manual instruction.

2.2.2 Estimation of osmotic adjustment by analyzing the pressure-volume curve

After the photosynthetic measurement, the leaf was excised with 5 leaflets attached. The excised leaf was rehydrated overnight with the cut trace in water under saturated humidity at 15ºC. Then the leaf was used for the pressure-volume (P-V) analysis according to Xu et al.[30]. The P-V curve was modeled as $-\Psi^{-1}=\{\Psi_{FT}^{-1}-\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{ap}]\}e^{-\alpha(1-\zeta)}+\pi_{s+a}^{-1}[\zeta_o-\beta(1-\zeta)-\zeta_{ap}]$. In the equation, Ψ was leaf water potential; π was osmotic potential; ζ was relative water content; subscripts FT, sym, ap and s+a mean full turgid, symplast, apoplast, and symplast + apoplast, respectively. The concentration of osmolytes (C_{osm}) was calculated as $C_{osm}=-410\pi$ [40,41].

2.2.3 Assessment of leaf water retention ability by analyzing the excised leaf transpiration declining curve

The leaf was excised under water, rehydrated overnight and then placed under light of 450 μmol/m^2·s. At 2-5 min intervals, fresh mass was recorded and the relative water content was calculated. A curve, with time (t) as abscissa and relative water content (ζ) as the ordinate, was modeled as $\zeta=[\zeta_0-\zeta_{SC}(1-\beta' t)]e^{-\alpha' t}+\zeta_{SC}(1-\beta' t)$, where $\beta' t$) and α' were constants. The subscripts 0 and SC mean "saturated" and "stomatal closed"[40]. The time used to theoretically dry up the leaf to its relative water content (τ) of 10% was calculated.

2.2.4 Determination of activities of nitrate reductase

The leaf samples were stored in a -85ºC freezer for use. The frozen sample was ground in liquid N in a chilled mortar. The extraction buffer contained 100 mM Tris-HCl at pH 7.5, 10 mM cysteine, 1 mM EDTA-Na, and 5 mM FAD. The buffer was added to the sample powder, homogenized again and then centrifuged at 15 000 × g for 30 min under 4ºC, with the supernatant used as enzyme sample. Nitrate reductase activity was measured by sulfanilamide spectrometry according to the manual of Sigma Aldrich (http://www.sigmaaldrich.com).

2.2.5 Analysis of the expression of nitrate reductase gene (NR1)

The expression of *NR1* gene was estimated with the real-time PCR system (Eco Real-Time PCR System, Illumina, San Diego, CA). The primer 5.0 was designed according to tomato Actin as the endogenous reference gene. The forward and reverse primers of the internal reference primer were S1 actin F: GGAATGGGACAGAAGGAT; S1 actin R: CAGTCAGGAGAACAGGGT with a product size 143 bp. The primers of *NR1* gene were showed as follows: Forward, (73-91bp) 18 bp 5' GGTTGAGGTGCTTGACTT 3' and Reverse, (233-215bp) 18 bp 5' CTCCCTTGTGAGGTTTGC 3' with a product size of 161bp. Extraction of total RNA was performed using RNeasy Plant Mini Kit (QIAGEN) according to the manual instruction.

2.2.6 Analysis of the expression of the stress-responsive gene (DREB2)

For isolation of *DREB2*cDNA from the tomato leaf, the primer was designed as Forward 5'-ATGATAATAATGTCTACAGAGCAA-3' — Reverse 5'-CTAATGTTGCCATAAAA AACTCTC-3'**.** The cDNA was used as template for PCR with primers as Forward 5'-ATGATAATAATGTCTACAGAGCAA-3' — Reverse 5'-CTAATGTTGCCATAAAAAACTCTC-3'). The specific primers, as Forward 5'-TGGCATCATACTTTCTACAATG-3' — Reverse: 5'-CTAATATCCTCGTCACATTTCAT-3', were used for RT-PCR amplification of actin gene. The details were described in Guo and Wang[30].

2.2.7 Measurement of superoxide anion (O_2^-), malondialdehyde (MDA) and Superoxide dismutases (SOD) activity in the tomato leaves

Superoxide anion (O_2^-) was measured according to Bissenbaev[32] with some modifications. Activity of superoxide dismutase (SOD) was measured by NBT (riboflavin-nitroblue tetrazolium) method[42,43]. Malondialdehyde (MDA) was

measured by thiobarbituric acid method[44,45].

2.2.8 Measurement of soil chemical properties

Chemical properties were measured using soil samples before fertilization, after nitrate salt application and after plant harvest. Electrical Conductivity (EC) was determined with an extract of soil : water = 1 : 5 by electrical conductivity meter (CT-57101B/CM-30G, Toa Co., Tokyo, Japan). The same soil extract was used to measure soil pH using a glass electrode (F-21, Horiba Co., Tokyo, Japan). The total carbon and total nitrogen were determined using an elemental analyzer (CN-Corder MT-700; Yanaco Co., Ltd., Kyoto, Japan). Concentrations of inorganic N and phosphorus were determined by colorimetry method using a photospectrometer (Hitachi U-2000 Tokyo, Japan). Other mineral nutrients were measured with atomic absorption spectroscopy (AA-6200, Shimazu, Kyoto, Japan).

2.3 Statistic analysis

The Data from all measurement in the present study were statistically analyzed based on Tukey's multiple comparisons using the software of DPS Data Processing System[46].

3 Results

3.1 Changes in EC

In Exp I, Wagner's pots each with 15 L of volume were used and the soil EC was less affected by irrigation compared with Exp II and Exp III, where small plastic pots were used (Table 1). Therefore, soil nutrient and EC were examined only in Exp I. One week after the treatment, nitrate salt application increased soil electrical conductivity (EC), which showed the soil salinity. It was reasonable that nitrate salt application increased total soil nitrogen concentration, inorganic N (NH_4^+ and NO_3^-) concentration and concentrations of K and Ca because these elements were included in the nitrate salts. However, application of microbial inoculant could not change EC and most of other parameters. After the plants were harvested, EC and the total and inorganic N got close without significant difference between treatments because of the plant uptake. K and Ca were remained more in salt application plots. There was no clear effect of MI application on the soil properties after plant harvested.

Table 1 Soil nutrients and chemical properties before fertilization, after salt application and after plant harvest (Ep. I)

Plot	pH	EC /mS·cm^{-1}	Total C /g·kg^{-1}	Total N /g·kg^{-1}	NH_4^+ /mg·kg^{-1}	NO_3^- /mg·kg^{-1}	P /mg·kg^{-1}	K /g·kg^{-1}	Ca g·kg^{-1}	Mg /cmol·kg^{-1}	CEC /cmol·kg^{-1}
Before fertilization											
Origin	5.89	0.044	37.10	2.70	0.65	0.57	43.6	144	2.048	0.248	18.3
After nitrate salt application											
CK	5.92	0.056b	48.3	3.86c	19.2b	29.3b	83.7	298b	2.712c	0.424	19.9
NS	6.05	0.069a	47.2	4.12a	23.2a	39.7a	79.3	419a	2.827b	0.433	19.2
MI	5.83	0.055b	47.6	3.93bc	20.2b	21.3c	81.7	312b	2.782b	0.421	19.7
S+I	5.82	0.071a	48.1	4.01b	25.8a	35.2a	82.7	427a	2.972a	0.442	19.8
NS	ns	**	ns	*	*	*	ns	**	*	ns	ns
MI	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns
S×M	ns	ns	ns	ns	*	*	ns	ns	ns	ns	ns
After plant harvested											
CK	5.86	0.052	40.9	3.48	4.53	13.7a	44.8a	163d	2.186b	0.415	18.1
NS	5.91	0.061	41.4	3.52	4.78	13.5a	42.1a	265b	2.420ab	0.404	18.4
MI	5.97	0.053	41.7	3.55	5.28	13.8a	39.4b	206c	2.324b	0.398	18.4
S+I	6.06	0.054	41.7	3.59	5.78	11.6b	37.3b	329a	2.523a	0.383	18.9
NS	ns	ns	ns	ns	ns	ns	ns	**	*	ns	ns
MI	ns	ns	ns	ns	ns	ns	*	**	ns	ns	ns
S×M	ns	ns	ns	ns	ns	*	ns	*	ns	ns	ns

3.2 Yield and quality of fruit and photosynthetic activities

3.2.1 Positive effect NS application at low rate on plants in larger pots (Exp I)

Application with nitrate salts (NS) of $Ca(NO_3)_2$ and KNO_3 in Exp I increased plant growth and fruit yield and also improved fruit quality by increasing glucose concentration (Table 2). Fruit yield improvement by NS application was attributed to the increase in fruit number rather than increased fruit size. NS application decreased photosynthetic capacity (P_C) but increased the maximum quantum yield (Y_Q). The effect of salt and can also be seen in the photosynthesis-light curves in Figure 1. The curve was much curvier in salt treatments than in the controls.

Table 2 Yield and quality of fruit and the photosynthesis activity parameters (Exp I)

Treat	Yield/g·plant^{-1}	Fruit number/F·plant^{-1}	Fruit size/g	Glucose/g·kg^{-1}	P_C/μmol·m^{-2}·s^{-1}	R_D/μmol·m^{-2}·s^{-1}	Y_Q/mol·mol^{-1}
CK	331c	5.6c	59.1b	12.1c	22.1b	3.2b	0.0471b
NS	383b	6.4b	59.9b	16.5b	20.2c	3.7a	0.0772a
MI	370b	6.1b	60.7a	23.5 a	23.6a	3.3b	0.0498b
S-MI	453a	7.3a	62.1a	24.7a	18.6d	3.7a	0.0787a
S	**	**	ns	*	*	*	**
MI	**	*	*	**	*	ns	ns
S×M	*	*	ns	ns	**	ns	ns

Note: P_C, photosynthetic capacity; R_D, dark respiration rate; Y_Q, quantum yield.

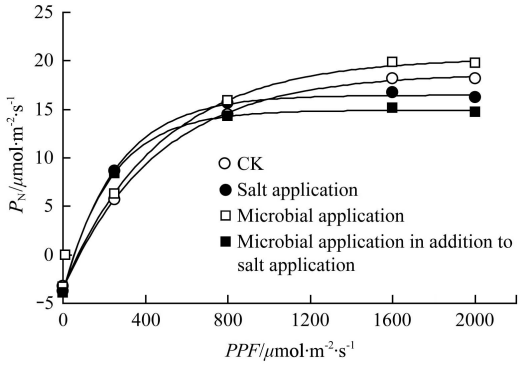

Figure 1 Photosynthesis-light response curve in leaves of tomato plants in different treatment plots in Exp I

3.2.2 Effect of NS application at low rate on plants in small pots (Exp II)

As shown in Table 3, application of NS in Exp II, where the small pot were used, could not result in positive effect on dry mass production as shown in Exp I although the application rate was the same. This might be due to the small soil volume that could not show enough soil buffer ability as in Exp I. Nevertheless, application of NS did not cause significant negative effect on dry mass production and photosynthetic activities.

3.2.3 Negative effect of NaCl application at low rate in small pots (Exp II)

At the same application rate as for NS, application of NaCl significantly decreased dry mass production and photosynthetic activities.

Table 3 Biomass production and photosynthetic activities in tomato plants grown in different conditions

Plot	Exp II						Exp III						Died plant/%
	Dry mass/ g·plant^{-1}	R/T	Leaf A/cm^2	P_C/ g·kg^{-1}	R_D/ μmol·m^{-2}·s^{-1}	Y_Q/ mol·mol^{-1}	Dry mass/ g·plant^{-1}	R/T	Leaf A/cm^2	P_C/ g·kg^{-1}	R_D/ μmol·m^{-2}·s^{-1}	Y_Q/ mol·mol^{-1}	
CK	0.345b	0.180d	151ab	13.1a	1.71b	0.0301c	0.332b	0.191	132b	11.7a	0.96c	0.0435c	0.0d
NS	0.339c	0.197c	144b	12.7a	1.85a	0.0334b	0.254c	0.174	101c	8.9b	1.47a	0.0376a	0.0d
CS	0.186f	0.225b	101c	10.3c	1.43d	0.0261d	0.161d	0.196	92d	9.3b	1.11b	0.0252c	46.7b
MI	0.361a	0.161e	159a	13.2a	1.74b	0.0292c	0.371a	0.187	153a	12.3a	1.01c	0.0355 b	2.9c
MN	0.327d	0.219b	139b	13.4a	1.81a	0.0367a	0.234b	0.182	93c	8.1c	0.90c	0.0249c	6.7c
MC	0.203e	0.276a	94c	11.2b	1.62c	0.0295c	0.131e	0.189	61e	7.2d	0.91c	0.0228c	60.2a
S	**	**	**	**	*	*	**	ns	**	**	**	**	**
MI	*	*	*	ns	ns	ns	*	ns	*	ns	ns	ns	ns
S×M	*	*	**	*	ns	*	*	ns	**	*	ns	*	*

3.2.4 Negative effect of applications of NS and NaCl at high rate in small pots in small pots (Exp III)

As shown by the results of Exp II in Table 3, NS application at high rate significantly decreased biomass and photosynthetic activities. At this high rate, application of NaCl showed negative effects more severely on biomass production and photosynthetic activities more severely than NS did. NaCl application also caused plant death at a rate as high as 46.7%.

3.2.5 Interaction of Microbial inoculant with NS or NaCl application on biomass production and photosynthetic activities (Exp I, II and III)

In Exp I, where the pot soil volume was larger, application of microbial inoculant (MI) increased fruit yield and improved fruit quality (Table 2). Fruit yield improvement by MI application was attributed to increased fruit size, while that by NS application was attributed to the increase in fruit number. There existed synergistic interaction between NS and MI applications on fruit yield. Photosynthetic capacity (P_C) was increased by applying MI although it was decreased by NS applications. MI application did not alleviate the photosynthetic depression caused by NS applications rather further depressed P_C in addition to NS application. In Exp II, MI application showed slight positive effect on biomass production but not on photosynthetic activities. IM application also showed a slight effect in alleviation of damage by NaCl application in both biomass production and photosynthetic activities. In Exp III, where the application rate of both NS and CS was doubled, MI application showed negative interaction with both NS and CS, aggravating instead of alleviating the damages.

3.3 Plant water relations and turgor maintenance at water-saturated status

3.3.1 Effects of NS and MI at low rate in large pots (Exp I)

Leaf water potential (Ψ_{FT}), osmotic potential (π_{FT}) and turgor potential (P_{FT}) indicated plant water relations without water stress when soil salinity was released. Both Ψ_{FT} and π_{FT} were lowered by NS application but π_{FT} was decreased more than Ψ_{FT} (Table 4). Therefore, P_{FT} was higher in plants with NS treatments because of $P_{FT} = \Psi_{FT} - \pi_{FT}$. This suggested that leaf turgor potential would be improved if the salinity was released after a period of treatment. Similarly, both leaf water potential at midday (Ψ_{MD}) and osmotic potential at midday (π_{MD}) were lower in NS plots but π_{MD} was lowered more than Ψ_{MD} and consequently leaf turgor potential at midday (P_{MD}) was a little higher in NS plots. This suggested that leaf turgor potential would be improved if the application rate of NS was moderately low. Application of MI aggravated effecting in decreasing leaf water potential both at midday and after stress released but helped maintain leaf turgor potential (Table 4).

3.3.2 Effects of NS and MI at low rate in small pots (Exp II)

In Exp II, application of both NS and CS decreased leaf water potential at both turgid status and midday. Because of more lowering in Ψ_{FT} than in π_{FT}, P_{FT} was higher in both NS and CS plots. However, at midday, because of more lowering in Ψ_{MD} than in π_{MD}, P_{MD} was lower in NS and CS plots. The positive effect of NS was larger than that of CS in maintenance of turgor potential. MI showed no direct effect on leaf water relations but showed interaction with both NS and CS in aggravating the salinity effect, causing further lower in P_{MD}.

3.3.3 Effects of NS and MI at high rate in small pots (Exp III)

In Exp III, where the application rate of both NS and CS was doubled, NS and CS significantly decreased leaf water potential at both turgid status and midday (Table 3). Because the salinity caused by both NS and CS could not induce further lowering in leaf osmotic potential, leaf turgor potential was lowered and reached zero at midday, causing plant death as shown in Table 3. MI showed aggravating effect on the salinity damage in aspects of leaf water relations.

Table 4 Variables from analysis of osmotic adjustment (Exp I)

Treat	Ψ_{FT}	π_{FT}	P_{FT}	Ψ_{MD}	π_{MD}	P_{MD}	π_{s+a}	π_{IP}	ζ_{IP}	ζ_{apo}	ζ_{sym}	α	β	C_{osm}	ΔC_{osm}
	MPa									%				osmol m^{-3}	
CK	–0.217a	–0.90a	0.68b	–0.712a	–0.915a	0.203d	–0.68a	–1.162a	0.883a	0.237a	0.763b	51.4a	0.981a	369.2d	0.0d
NS	–0.232b	–1.01b	0.78a	–0.757b	–1.041b	0.284b	–0.82bc	–1.251b	0.859b	0.185b	0.815a	29.9c	0.919b	412.8b	43.7b
MI	–0.225b	–0.94a	0.71b	–0.702a	–0.918a	0.216c	–0.78b	–1.193a	0.795c	0.218a	0.782b	43.7b	0.922b	395.8c	16.4c
MN	–0.267c	–1.06c	0.79a	–0.793c	–1.112c	0.319a	–0.87c	–1.486c	0.849b	0.178b	0.828a	26.0c	0.907c	432.5a	63.3a
S	*	**	*	*	*	*	**	*	*	**	**	**	*	**	**
MI	*	*	*	ns	ns	*	*	ns	*	ns	ns	*	*	*	*
S×M	*	*	ns	*	*	**	*	**	ns	*	*	*	*	*	*

Note: Ψ_{FT}, π_{FT} and P_{FT} were leaf water potential (Ψ), osmotic potential (π) and turgor potential (P) at full turgid status. π_{s+a} was $\pi$when symplastic solution was diluted by apoplastic solution. π_{IP} was π at incipient plasmolysis. ζ_{IP} was the relative leaf water content (ζ) at incipient plasmolysis. ζ_{apo} and ζ_{sym} was ζ in fractions of apoplasm and symplasm. The coefficients α and β were related with the curvature of first steep part and the second sloping part in the P-V curve. C_{osm} was the osmotic concentration and ΔC_{osm} was the active increase in C_{osm} due to the osmotic adjustment.

3.4 Osmotic adjustment

3.4.1 Effects of NS and MI at low rate in large pots (Exp I)

The ability of osmotic adjustment can be shown by the active increase in solute concentration. In Exp I, with the control plot (CK) as the zero reference ($\Delta C_{osm} = 0$), osmotic concentration at fully turgid status (ΔC_{osm}) was 43.9 and 63.3 osmol/m^3 in leaves of NS treatments with and without MI application, respectively (Table 4). The results suggested that osmotic adjustment really occurred when NS was applied at a moderately low rate. The osmotic adjustment caused by MI application was small with an additive interaction between NS and MI applications.

3.4.2 Effects of NS and MI at low rate in small pots (Exp II)

Application of both NS and CS induced additional active solute accumulation but this positive effect by NS was larger than that by CS (Table 5). MI showed no effect on additional active solute accumulation but showed synergistic interaction with NS and CS.

3.4.3 Effects of NS and MI at high rate in small pots (Exp III)

In Exp III (Table 6), where the application rate of NS and SC was doubled, application of NS showed a little effect in increasing solutes but this positive effect was much smaller than in Exp I and Exp II. Different from results in Exp I and Exp II, application of CS did not induce additional active accumulation of solutes. MI showed no direct effect on active solute accumulation neither the interaction effect with NS and CS.

Table 5 Variables from analysis of osmotic adjustment (Exp II)

Treat	Ψ_{FT}	π_{FT}	P_{FT}	Ψ_{MD}	π_{MD}	P_{MD}	π_{s+a}	π_{IP}	ζ_{IP}	ζ_{apo}	ζ_{sym}	α	β	C_{osm}	ΔC_{osm}
	MPa									%				osmol m^{-3}	
CK	–0.198a	–0.745a	0.548c	–0.806a	–1.145b	0.339a	–0.722a	–1.178	0.896a	0.228a	0.772b	55.2a	0.98	305.5e	0.0e
NS	–0.212c	–0.902c	0.690a	–0.875b	–1.184c	0.309b	–0.836d	–1.292	0.876b	0.198b	0.802a	43.0b	0.97	369.7b	64.1b
CS	–0.206b	–0.819b	0.613b	–0.973c	–1.164c	0.191d	–0.779c	–1.245	0.886a	0.204b	0.796a	45.0b	0.97	335.7c	30.2b
MI	–0.195a	–0.773a	0.578c	–0.801a	–1.136b	0.335a	–0.741b	–1.207	0.865c	0.240a	0.760b	56.0a	0.98	316.8d	11.3d
NM	–0.216c	–0.929d	0.714a	–0.884b	–1.109a	0.225c	–0.846e	–1.273	0.876b	0.193b	0.807a	44.9b	0.98	381.0a	75.4a
CM	–0.226d	–0.837b	0.611b	–1.021d	–1.192d	0.171f	–0.827d	–1.226	0.876b	0.199b	0.801a	45.7b	0.97	343.3c	37.7c
S	*	**	*	*	*	*	**	*	*	**	**	**	ns	**	**
MI	ns	ns	ns	ns	ns	ns	*	ns	*	ns	ns	ns	ns	ns	ns
S×M	*	*	ns	*	*	*	*	**	ns	*	*	*	ns	*	*

Table 6 Variables from analysis of osmotic adjustment (Exp III)

Treat	Ψ_{FT}	π_{FT}	P_{FT}	Ψ_{MD}	π_{MD}	P_{MD}	π_{s+a}	π_{IP}	ζ_{IP}	ζ_{apo}	ζ_{sym}	α	β	C_{osm}	ΔC_{osm}
	MPa									%				osmol m^{-3}	
CK	–0.203a	–0.792a	0.589a	–0.862a	–1.126	0.264a	–0.743a	–1.193	0.861	0.263a	0.737c	57.2a	0.97	324.7b	0.0b
NS	–0.285b	–0.862b	0.577b	–1.075b	–1.107	0.032b	–0.796b	–1.216	0.855	0.229c	0.771a	46.0b	0.98	353.4a	28.7a
CS	–0.265c	–0.798a	0.533c	–1.179b	–1.176	0.000b	–0.792b	–1.234	0.858	0.244b	0.756b	48.0b	0.97	327.2b	2.5b
MI	–0.201a	–0.796a	0.595a	–0.881a	–1.127	0.246a	–0.763a	–1.187	0.852	0.254a	0.746c	56.3a	0.97	326.4b	1.6b
NM	–0.297a	–0.809a	0.512c	–1.164b	–1.009	0.000b	–0.855c	–1.203	0.854	0.226c	0.774a	46.8b	0.98	331.7b	7.0b
CM	–0.286b	–0.802a	0.516c	–1.176b	–1.162	0.000b	–0.866c	–1.207	0.857	0.249b	0.751b	47.2b	0.97	328.8b	4.1b
S	*	*	*	*	ns	*	**	ns	ns	*	*	*	ns	*	*
MI	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S×M	*	ns	*	*	ns	*	*	ns	ns	ns	ns	ns	ns	ns	ns

3.5 Water stress tolerance shown by osmotic potential and relative water content at incipient plasmolysis

3.5.1 Effects of NS and MI at low rate in large pots (Exp I)

Water stress tolerance can be shown as one of the indicators by osmotic potential (π_{IP}) or relative water content (ζ_{IP}) at incipient plasmolysis, when the cell membrane is just separated from the cell wall or when the leaf turgor potential is zero. In Exp I, where the application rate of NS was low and the pots were large, π_{IP} was lowered by NS application although ζ_{IP} was not (Table 4). This suggested that a leaf in NS treatment could maintain its turgor to a

more severe water stress level in comparison with its control plots. MI application did not show this kind of effect but showed an additive or synergistic interaction with NS treatment.

3.5.2 Effects of NS and MI at low rate in small pots (Exp II)

In Exp II, where the pots were smaller than in Exp I, application of NS lowered both π_{IP} and ζ_{IP}. CS also lowered π_{IP} but ζ_{IP}. Although the positive effect of NS and CS was lower than in Exp I, the improvement in water stress tolerance by NS and CS application was confirmed. MI showed no significant direct effect on π_{IP} but significant on ζ_{IP}, with significant negative interaction on π_{IP} with NS and CS.

3.5.3 Effects of NS and MI at high rate in small pots (Exp III)

In Exp III, where the application rate was doubled and severe salinity damages were induced, application of both NS and CS did not show any effect on π_{IP} and ζ_{IP}.

3.6 Cell water re-compartmentation

3.6.1 Effects of NS and MI at low rate in large pots (Exp I)

Usually, water in a cell of tomato leaf is compartmented 0.76% in the symplasm, called symplastic water fraction (ζ_{sym}), and 24% in cell wall or apoplasm, called the apoplast water fraction (ζ_{sym}), as shown in the control plot of Exp I (Table 4). When the plant meets water or salinity stress, water in cell wall or the apoplasm may moves into the symplasm. Thus, water fraction would increase in symplasm and decrease in apoplasm, no matter the change in absolute value of the total cell water content was large or small. In Exp I, ζ_{sym} was increased and the apoplast water fraction (ζ_{sym}) decreased by NS application. MI application did not change the cell water fraction but showed a synergistic interaction with NS treatment.

3.6.2 Effects of NS and MI at low rate in small pots (Exp II)

In Exp II, NS application increased symplastic water fraction more than CS did. MI application did not significantly affect the cell water compartmentation but showed a synergistic interaction with NS and CS.

3.6.3 Effects of NS and MI at high rate in small pots (Exp III)

In Exp III, NS application increased cell symplastic water fraction but CS did not reach the significant levels. MI did not show effect on cell water compartmentation and showed neither interaction with NS or CS.

3.7 Nitrate content, nitrate reductase and the expression of nitrate reductase gene

3.7.1 Effects of NS and MI at low rate in large pots (Exp I)

The total leaf nitrogen content was increased by NS application and this was proportional to leaf color at both early and late stages. MI application did not increase the total nitrogen content and leaf color but showed a synergistic interaction with NS treatment (Table 7). Leaf nitrate concentration was not higher or even lower in NS treatments than the controls and this was not consistent with the total leaf nitrogen content. The lower nitrate concentration might be attributed to the higher activity of nitrate reductase in NS treatments than in the controls. MI application did not show much effect on nitrate concentration and the activity of nitrate reductase. The relative expression of NR gene was higher in NS treatments and this was consistent with the nitrate reductase activity. MI application also showed a promoting effect on NR gene expression with additive or synergistic interaction between NS and MI applications.

3.7.2 Effects of NS and MI at low rate in small pots (Exp II)

In Exp II, NS application increased leaf total N and nitrate contents but CS decreased N (Table 8). MI did not show any effect on leaf total N and nitrate content. Leaf color showed a consistent changing trend with the leaf N status. NS increased nitrate reductase (NR) activity but CS decreased NR activity. MI increased NR activity and showed a positive interaction with NS and CS. NS application induced up-regulation expression of *NRI* gene but did not show this effect. MI did not affect the *NRI* expression but showed a significant positive interaction with NS and CS.

3.7.3 Effects of NS and MI at high rate in small pots (Exp III)

In Exp III, NS increased but CS decreased the total leaf N and nitrate N content and MI did not affect leaf nitrogen (Table 9). Leaf color reflected the leaf N status. NS induced up-regulation of *NRI* gene and increased NR activity. CS did not show this effect and MI also showed no this effect.

Table 7 Nitrogen contents, nitrate reductase activity and relative expression of NR gene in tomato leaves in different plots (Exp I)

Plot	Total N /g kg^{-1}DM	Nitrate /g kg^{-1}FM	Leaf color	NR activity/mg kg^{-1}·h^{-1}			*NR1* expression		
				1 d	7 d	35 d	1 d	7 d	35 d
CK	14.7b	1.03b	38.4c	0.77ab	0.12c	0.59d	0.81c	2.01c	3.34c
NS	15.7a	1.21a	48.2a	0.79ab	1.58a	1.58b	1.34b	4.704b	6.06b
MI	15.0b	1.12ab	37.0c	0.69b	0.29c	1.02c	1.47b	2.11c	7.59b
MNI	15.5a	0.74c	43.5b	0.85a	1.17b	2.31c	6.07a	8.79a	12.33a
S	*	*	**	ns	**	**	**	**	**
MI	*	*	ns	ns	ns	*	**	ns	**
S×M	*	**	*	*	ns	*	**	**	*

Table 8 Nitrogen contents, nitrate reductase activity and relative expression of NR gene in tomato leaves in different plots (Exp II)

Plot	Total N /g·kg^{-1} DM	Nitrate /g·kg^{-1} FM	Leaf color	NR activity/mg·kg^{-1}·h^{-1}			*NR1* expression		
				3 d	8 d	15 d	3 d	8 d	15 d
CK	13.2b	1.14b	34.1bc	1.48d	0.90c	0.60d	1.00c	2.18c	3.70c
NS	14.4a	1.33a	35.3b	1.95b	1.55b	2.38a	15.50b	11.40b	6.06b
CS	12.1c	1.01c	33.6c	1.39d	0.80c	1.22b	1.22c	2.71c	4.59c
MI	13.4b	1.17b	35.0b	2.04b	0.79c	1.46b	5.76c	3.07c	4.33c
MN	14.9a	1.39a	38.1a	3.28a	3.33a	2.54a	22.70a	17.74a	12.30a
MC	11.8c	1.09c	34.4b	1.71c	1.79b	1.62b	2.36c	2.96c	2.39c
S	*	*	*	*	**	**	**	**	**
MI	ns	ns	ns	*	ns	*	ns	ns	ns
S×M	*	*	*	*	*	ns	**	**	*

Table 9 Nitrogen contents, nitrate reductase activity and relative expression of NR gene in tomato leaves in different plots (Exp III)

Plot	Total N /g kg^{-1} DM	Nitrate /g kg^{-1} FM	Leaf color	NR activity/mg·kg^{-1}·h^{-1}			*NR1* expression		
				6 d	7 d	31 d	6 d	7 d	31 d
CK	12.2b	0.93c	31.6a	1.39a	1.45b	0.98b	1.02b	0.98c	1.03c
NS	13.7a	1.12a	36.5a	1.72a	2.25a	1.92a	1.27a	1.90b	2.19b
CS	10.9c	0.84d	25.9b	1.58a	1.65b	0.99b	0.87c	0.92c	0.85c
MI	12.5b	0.97c	33.7a	1.45a	1.64b	1.18b	0.87c	0.93c	1.02c
MN	14.1a	1.09b	37.5a	0.95b	1.03c	0.87b	1.49a	2.72a	3.26a
MC	10.2c	0.79d	28.4b	0.88b	0.69d	0.54c	0.92c	1.02c	0.96c
S	*	*	*	*	**	**	**	**	**
MI	ns	ns	ns	*	ns	*	ns	ns	ns
S×M	*	*	*	*	*	ns	**	**	*

3.8 Antioxidation activity

3.8.1 Effects of NS and MI at low rate in large pots (Exp I)

NS application increased O_2^- concentration in tomato leaves at the time of one day after treatments started (Table 10). However, at this time, NS application had not induced the activation of SOD, which responded to breakdown the stress-induced O_2^-. Therefore, the concentration of MDA increased, which suggested that the cell membrane was damaged by the salinity stress. This was consistent with the midday wilting of the tomato that was observed in situ several hours after NS was applied. However, one week after treatments started, both O_2^- and MDA in tomato leaves in NS plot showed similar levels to those in control plot but the activity of SOD got higher than in control plots, especially in the plot of NS plus MI. This suggested that tomato plants acclimated to NS salinity by increasing SOD activity. MI application induced activation of SOD one day after treatment started and therefore there was no increase in O_2^- and MDA at this time and 7 days later. Because there was no O_2^- increase caused by MI application from the beginning, there was no more activation of SOD 7 days after treatments started. However, MI application showed high synergistic interaction with NS application.

3.8.2 Effects of NS and MI at low rate in small pots (Exp II)

In Exp II, NS application did not induce O_2^-increase but did 8 days after treatment started and CS showed this effect from Day 3 (Table 11). Both NS and CS increased MDA concentration. NS and CS both induced up-regulation expression of the stress-response gene (*DREB2*) and increased SOD activity through the whole treatment period. MI application showed no effect on the gene expression, SOD activity and concentration of both O_2^- and MDA but MI showed interactions with NS and CS on cases.

3.8.3 Effects of NS and MI at high rate in small pots (Exp III)

In Exp III, where application rate of NS and CS was doubled, both NS and CS increased O_2^- concentration immediately after treatments started and the high concentration of O_2^- maintained through the whole experimental period but lowered down at the end for CS treatment (Table 12). MI showed no direct effect on O_2^- concentration but synergistically interacted with NS and CS. MDA concentration did not show clear changing trends affected by NS, CS and MI. Both NS and CS decreased SOD activity although they did induce up-regulation expression of *NRI* gene. MI also showed no clear effect on SOD but showed interactive effect with NS and CS on *NRI* expression.

Table 10 SOD activity and concentrations of O_2^- and MDA as well as the relative expression of the stress-responsive gene of *DREB2* (Exp I)

Plot	O_2^-/mmol·kg^{-1} FM			MDA/mmol·kg^{-1} FM			SOD/U·g^{-1} FM			*DREB2* expression		
	1 d	7 d	35 d	1 d	7 d	35 d	1 d	7 d	35 d	1 d	7 d	35 d
CK	15.2d	16.8	13.2	1.01b	1.12	0.87	1980b	2040c	1324c	1.07c	1.06b	1.21b
NS	37.2b	17.0	15.9	1.24a	1.13	0.79	1740b	2760b	1763b	1.84b	3.74a	7.37a
MI	29.1c	14.6	16.1	0.97b	0.97	0.91	2280a	2100c	1401c	1.01c	1.23b	1.80b
MN	47.8a	17.3	16.4	1.26a	1.15	1.01	2440a	4904a	2352a	2.24a	4.42a	9.07a
S	**	ns	ns	*	ns	ns	ns	**	*	*	*	**
MI	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S×M	*	ns	ns	ns	ns	ns	*	**	**	**	**	*

Table 11 SOD activity and concentrations of O_2^- and MDA as well as the relative expression of the stress-responsive gene of *DREB2* (Exp II)

Plot	O_2^-/mmol·kg^{-1} FM			MDA/mmol·kg^{-1} FM			SOD/U·g^{-1} FM			*DREB2* expression		
	3 d	8 d	15 d	3 d	8 d	15 d	3 d	8 d	15 d	3 d	8 d	15 d
CK	3.27b	4.98c	1.34c	0.69c	0.83c	0.55d	402d	897c	371c	0.37d	1.01d	19.71d
NS	3.02b	8.16a	2.25c	1.04b	1.35b	1.81b	521c	990c	527b	0.48c	1.67c	17.25d
CS	5.59a	10.4a	8.76a	1.42a	2.34a	2.31a	742b	788c	576b	0.61b	2.24b	34.80b
MI	2.86b	4.90c	3.39c	0.90bc	1.18bc	0.66d	347d	742c	346c	0.42cd	1.24cd	19.24d
MN	3.87b	9.09a	5.94b	0.87bc	2.07a	1.08c	765b	1787a	776a	0.65b	2.01b	25.11c
MC	4.68a	6.70b	6.10b	1.16b	1.86a	1.41b	882a	1237b	812a	2.06a	2.84a	48.52a
S	**	**	**	*	**	**	**	ns	*	*	**	*
MI	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S×M	*	**	*	ns	ns	ns	*	**	*	*	ns	**

Table 12 SOD activity and concentrations of O_2^- and MDA as well as the relative expression of the stress-responsive gene of *DREB2* (Exp III)

Plot	O_2^-/mmol·kg^{-1} FM			MDA/mmol·kg^{-1} FM			SOD/U·g^{-1} FM			*DREB2* expression		
	6 h	7 d	31 d	6 h	7 d	31 d	6 h	7 d	31 d	6 h	7 d	31 d
CK	2.16c	6.32c	5.52b	2.31	1.51d	1.19c	946a	707a	583a	1.00c	1.00d	1.00c
NS	4.64b	8.24b	8.32a	2.42	1.72c	1.74b	749b	544b	473b	1.32b	1.69b	1.81b
CS	4.88b	8.08b	4.24b	1.34	1.88ab	0.79d	697b	435c	366c	1.21b	1.46c	2.03b
MI	2.64c	7.40bc	3.60b	1.91	1.61cd	1.71b	809a	595b	603a	0.97c	1.07d	1.12c
MN	5.36b	11.04a	9.36a	2.77	1.78b	1.1c	704b	453c	374c	1.45a	1.63b	2.72a
MC	7.92a	10.32a	5.52b	2.55	1.98a	2.22a	574c	364d	366c	1.63a	1.94a	2.96a
S	**	*	*	ns	*	*	*	*	*	*	*	*
MI	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
S×M	*	*	ns	ns	ns	*	ns	*	ns	*	*	*

4 Discussion

Excessive use of nitrate fertilizers has caused big problems in greenhouse soils in China[13,14]. The main problem is the secondary soil salinization. This is mainly attributed to the increasing demand of growers for higher and higher yielding. It is not denying that fertilizers, especially the nitrogen fertilizers are necessary for high crop yield and a moderately high soil EC benefits high quality of fruits such as tomatoes[47]. Research has shown that greenhouse tomato fruit quality has been improved by a moderately high EC in the nutrient solution without yield reduction by applying higher EC solution when the evapotranspiration demand is lower in the night and lower EC solution when the evapotranspiration demand is high at midday[48]. Health of crops can also be improved by stimulation with moderately high EC in the nutrient solution and this is called one of the applications of xerophytophysiology and signal transduction to plant production[49]. However, use of high EC is operated in hydroponic conditions without leaching of pollutants to the environment. In the soil-based greenhouses, the situation is different. Excessive fertilization in the soil-based greenhouses may cause many problems, such as accumulation of salts and the consequent salinization of the soil, pollutants leaching to the underground and surface waters[15,50]. Applications of organic and microbial materials have been tried in reducing the soil salinity [39] caused by high content of sodium. However, it is needed to know whether or not applications of a microbial inoculant can reduce the damage caused by soil salinization from the excessive application of nitrogen fertilizers. Therefore, in Exp I of the present study, nitrate salts (NS) of $Ca(NO_3)_2$ and KNO_3 was applied to potted tomato plants to simulate the soil salinity caused by excessive fertilization. The pot volume, with a soil surface of 0.05 m^2 and a height of 0.3 m, was large enough to supporting tomato fruiting. A microbial inoculant was used and expected to alleviate the damage caused by the soil salinity. One day after the treatment started, soil salinity caused by NS application really decreased the leaf water potential and leaf turgor potential at midday, induced the increase in superoxide radicals and cell membrane damage shown by high MDA concentration. However, as the treatment was prolonged for one week, activation of SOD was induced and consequently concentrations of O_2^- and MDA were lowered to the normal levels. Three weeks after the treatments started, P-V curve was analyzed and the results showed that osmotic adjustment was induced and leaf turgor potential at no water stress was improved. The active increase in the concentration of osmolytes in addition to the turgor improvement suggested that osmotic adjustment surely occurred in response to soil salinity. The cell water fraction in symplasm was increased and that in apoplasm was reduced and this adjustment was favorable for the biochemical metabolism in the symplasm[51]. The osmotic potential at incipient plasmolysis or at zero turgor was lower in leaves of NS treated tomato plants than in the control. This suggested that NS application increased the water stress tolerance and maintained leaf turgor to severer stress level. As the treatment of NS application prolonged for weeks, up-regulation of the nitrate reductase (NR) gene and the consequent activation of nitrate reductase were induced. Therefore, despite the high total nitrogen content and deep leaf color, nitrate concentration was reduced as the NS treatment prolonged. The abovementioned results suggested that the tomato plants acclimated to the prolonged NS treatment at both physiological and molecular biological levels. As the nitrate was a necessary nutrient for tomato plants and the tomato plants acclimated to the soil salinity caused by NS treatment, fruit yield and total biomass production were still higher in NS treatment than in the control, despite the salinity stress and damage at the beginning. This is also the difference between salinities caused by nitrate salts and non-nitrate salts in the effect on plant production. In the successive studies with Exp II and Exp II, the same salinity as in Exp I and further severer salinity caused by nitrate salts and NaCl salt were designed. Comparison between salinities caused by nitrate salts and non-nitrate salts were made. Even at the same salinity level, salinity caused by NaCl could not show positive effect as nitrate salts did although both induced up-regulation expression of the stress-responsive gene and the consequent osmotic adjustment and activation of antioxidant enzymes. In addition to the positive osmotic adjustment, nitrate at the moderately high level improved biomass production. Even at the low applying rate, NaCl could not show positive effect on biomass production and consequently the osmotic adjustment ability was lower than that caused by nitrate salts because the osmotic adjustment should be a process consuming biomass and energy. In Exp III, where applying rate was doubled, NS did not show as much positive effect as that at low rate. NaCl salt at doubled applying rate salt showed worse effect on plant growth without real positive adaptation at both molecular and physiological levels. Even at the low applying level, NS salinity showed more positive effect on tomato plants in large pots than on those in small pots. The large volume might help tomato plant to better adapt to the salinity stress and favorite the stress-responsive regulations. Application of microbial inoculant was expected to alleviate the salinity damage no matter itself could or not show positive effect on the tomato crop. Actually, microbial inoculant

application itself also improved the tomato crop yield and fruit quality by increasing the photosynthetic activities. Clarification of the detailed mechanisms for microbial inoculant effect in crop improvement was not planned in the present study and will be considered in the future studies. Application of microbial inoculant did not reduce the soil salinity shown by higher EC levels but did alleviate the salinity damage and synergistically increased the xerophytophysiological regulation caused by the soil salinity in addition to its positive effects on the tomato crop. There is also other research case that application of microorganisms cannot reduce soil salinity directly but alleviate salinity damage to the crops [51]. The microbial inoculant used in the present study contains lactic bacteria, yeasts, actinomycetes, photosynthetic bacteria as the main components with a pH of 3.5 in the original solution and it is different from other cases in both strain components and the chemical and physical properties of the final inoculant product. Further studies are needed to clarify how and what kinds of components in the inoculant contributed to the positive effects on the tomato plants under soil salinity caused by excessive application of nitrate fertilizers.

5 Conclusions

The following conclusions were drawn based on the results in the present study.

1) Application of nitrate salts at low rate induced salinity damage to potted tomato plants immediately after the treatment started. The tomato plants acclimated to the salinity stress by up-regulation expression of stress-responsive genes and the consequent xerophytophysiological regulations such osmotic adjustment and by activating antioxidant enzymes, nitrate reductase gene and the homological enzyme. As a necessary nutrient and cooperated with the xerophytophysiological regulations, nitrate salts at the low rate increased tomato fruit yield and the total plant biomass production despite of salinity stress.

2) However, salinity caused by application of NaCl did not show positive effect on tomato plants as nitrate salts did, although NaCl, at low applying rate, also induce up-regulation of stress-responsive genes and xerophytophysiological regulations.

3) As the applying rate was doubled, nitrate salts did not show positive effect and NaCl showed severe damage effect on tomato plants.

4) Application of microbial inoculant alleviated damages at low salinity level but aggravated damages at high salinity level irrespective of its own positive effects to the tomato plants. Mechanisms for the effect of microbial inoculant need to be clarified in further studies.

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