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Fulvic Acid Alleviates the Toxicity Induced by Calcium Nitrate Stress by Regulating Antioxidant and Photosynthetic Capacities and Nitrate Accumulation in Chinese Flowering Cabbage Seedlings

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Abstract: Continuous cropping can lead to an excessive accumulation of nitrate in facility-cultured soil. Excessive accumulation of nitrate gradually becomes the main reason for crop failure in vegetables and endangers human health. Therefore, the exploration of effective measures to decrease abundant nitrate accumulation in Chinese flowering cabbage is indispensable. In this study, a kind of plant growth regulator, fulvic acid (FA), was used to study its positive effect on alleviating the growth inhibition induced by excessive $Ca(NO_3)_2$ in Chinese flowering cabbage. Meanwhile, we conducted hydroponic cultivation and measured the growth indices, photosynthetic and oxidation-reduction characteristics of Chinese flowering cabbage with different treatments. After determining the optimal treatment concentration, we mainly designed four treatment groups, including Con, FA, $Ca(NO_3)_2$ and FA + $Ca(NO_3)_2$ cotreatment, to explore the regulatory mechanism by which FA alleviates $Ca(NO_3)_2$ stress in Chinese flowering cabbage. The results showed that FA can effectively alleviate the inhibitory effect of excessive $Ca(NO_3)_2$ on the growth of Chinese flowering cabbage seedlings. FA recovered the photosynthetic capacity of seedlings under $Ca(NO_3)_2$ stress. In addition, FA depressed the accumulation of $O^{2\cdot-}$, H_2O_2, malondialdehyde (MDA) and relative electrical conductivity, but increased the activity of antioxidant enzymes, including SOD, POD, CAT and APX, which finally enhanced the stress resistance of Chinese flowering cabbage to $Ca(NO_3)_2$. The expression of nitrate-related transporters, *BcNRT1.1* and *BcNRT1.5*, was depressed by FA, which inhibited redundant nitrate absorption and restricted more nitrate from being stored in the roots instead of being transferred to the shoot. Ultimately, nitrate accumulation in the edible part was reduced in Chinese flowering cabbage seedlings. In general, exogenous FA may alleviate nitrate stress by improving oxidation resistance, photosynthetic capacity and redundant $Ca(NO_3)_2$ accumulation in Chinese flowering cabbage.

Keywords: calcium nitrate; Chinese flowering cabbage; fulvic acid; photosystem; antioxidant capacity; nitrate accumulation

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1. Introduction

Facility agriculture has protected crop production from seasonal barriers [1–3]. Calcium nitrate ($Ca(NO_3)_2$) is regularly chosen as a primary chemical fertilizer and standard formulation of hydroponic nutrient solution due to its water-soluble property [4]. However, with the rapid development of facility agriculture, secondary salinization induced by

$Ca(NO_3)_2$ in continuous cropping soil has become increasingly serious due to excessive fertilization and intensive farming [5]. The excessive $Ca(NO_3)_2$ content in the soil could cause serious damage to both the yield and quality of vegetables. The superfluous $Ca(NO_3)_2$ accumulated in vegetables may lead to oxidative damage and metabolic disturbances, which finally results in crop failure in Chinese cabbage, cucumber and tomato [2,5,6]. Several studies have indicated that vegetables contribute to the main source of nitrate in humans. Moreover, the excessive nitrate can convert to nitrite, which synthesizes carcinogens and induces cancer in the digestive system [7]. Therefore, alleviating $Ca(NO_3)_2$ stress and reducing the nitrate accumulation in edible parts of vegetables are critical.

Different methods have been used to reduce excessive nitrate accumulation in vegetables including soil reclamation, light environment regulation, reducing the application of nitrogen fertilizer and using organic nitrogen instead. However, the availability of these methods is influenced by several factors, including time consumption, low efficiency and crop failure [8–10]. Therefore, selecting a more effective and low-cost method to reduce the redundant accumulation of nitrate is necessary to improve the production and nutritional quality of vegetables. Fulvic acid (FA), an active humic substance, has high polarity, hydrophilic radicals and active organic functional groups [11]. In humic acid, FA is an ideal organic component of low molecular weight and has higher solubility than other substances of humic acid at lower pH [12,13]. Many studies have revealed that FA, as a growth regulator, could improve the growth of plants by regulating their photosynthetic capacity and the level of endogenous hormones [11,14]. In detail, the application of FA could reliably increase the yield and quality of spring wheat and sugar beet [14]. In addition, FA has the capacity to regulate several secondary metabolisms including starch and sucrose metabolism, which markedly enhanced the drought resistance of tea plants [11].

Chinese flowering cabbage, *Brassica campestris* L. ssp. *Chinese*, is an important vegetable crop in Chinese facility cultivation. However, the yield and quality of Chinese flowering cabbage have been significantly reduced by excessive $Ca(NO_3)_2$ in continuous cropping soil [5,15]. In this study, the effects of FA on the growth of seedlings, the photosystem, the antioxidant system and nitrate accumulation in Chinese flowering cabbage were evaluated. These results may contribute to understanding the regulatory mechanism of FA-alleviated $Ca(NO_3)_2$ stress and further reveal the physiological functions of FA in vegetables.

2. Materials and Methods

2.1. Plant Material and $Ca(NO_3)_2$ Treatments

'Jin Qiuhong 2', *Brassica campestris* L. ssp. *Chinensis*, was used as the research material. The seeds were sterilized with 75% alcohol and 1% (v/v) NaClO before being soaked in deionized water at room temperature for 3–5 h. After overnight germination, the germinated seeds were cultured with 1/4 Hoagland's solution and placed in a light incubator. The temperature and photoperiod in the incubator were controlled with 23–25 °C and 16/8 h light/dark cycles, respectively. We used 1 mM HCl or 1 mM NaOH to keep the pH of the nutrient solution at 6.5 throughout. The uniform seedlings were selected to conduct the following experiments after being grown to two true leaves and a terminal bud. Seedlings grown in 1/4 Hoagland's solution (with 15 mM $Ca(NO_3)_2$) were used as the control and described as Con. All the treatments were described as follows: (I) Con [15 mM $Ca(NO_3)_2$]; (II) 80 mM $Ca(NO_3)_2$ + 40 µM FA; (III) 80 mM $Ca(NO_3)_2$ + 60 µM FA; (IV) 80 mM $Ca(NO_3)_2$ + 80 µM FA; (V) 80 mM $Ca(NO_3)_2$ + 100 µM FA; (VI) 80 mM $Ca(NO_3)_2$ + 120 µM FA; and (VII) 80 mM $Ca(NO_3)_2$ + 150 µM FA. After 5 days of different treatments, 3 replicates with 20 plants per replicate were used to determine the growth indices such as plant height, root length, stem, root stem and fresh weight. A ruler (0.1 cm) was used to measure plant height and root length, and an electronic balance (0.0001 g) was used to detect the dry weight and fresh weight of the plant samples.

2.2. Measurements of Photosynthetic Parameters

The true leaf was used for calculating the net photosynthetic rate, transpiration rate, and stomatal conductance, and for gas exchange determination using a portable photosynthetic apparatus (LI-6800, LI-COR Biosciences Inc., Lincoln, NE, USA). Three replicates of 10 plants each were performed in each treatment.

2.3. Analysis of Malondialdehyde (MDA) Concentration and the Relative Electrical Conductivity of Leaves

The MDA concentration was measured using the thiobarbituric acid method [5]. Fresh leaves (0.5 g) were homogenized with 5 mM 5% trichloroacetic acid (TCA) and then centrifuged at 5000 rpm for 15 min. After that, the supernatant was thoroughly blended with 0.67% TCA in a boiling water bath. The absorbance was acquired at 450, 532 and 600 nm. The concentration of MDA was calculated using the formula C (μmol/L) = 6.45 (A532 − A600) − 0.56A450.

Fresh seedlings were vacuumized to completely sink the blade in deionized water. Then, a conductivity meter was applied to detect the conductivity of the solution, which was represented by A1. After boiling the leaves in water for 15 min, the conductivity of the solution was detected using a conductivity meter [5], which was represented by A2. The following formula, A1/A2 × 100%, was applied to measure the relative conductivity.

2.4. Histochemical Detection and Content Analyses of Hydrogen Peroxide (H_2O_2) and Superoxide Radical ($O^{2\cdot-}$)

The 3,3′-diaminobenzidine (DAB) staining method was applied to measure H_2O_2 production by staining the leaves with 0.1% DAB solution for 2 h [5,16]. The nitro blue tetrazolium (NBT) staining method was used to detect the $O^{2\cdot-}$ production in leaves by observing the purple-blue color [17]. All the samples were decolorized using 95% ethanol for 24 h at 37 °C to remove the chlorophyll or superfluous dye. A light microscope was used to observe the decolorized leaves (model: Stemi 2000-C; CarlZeiss, Jena, Germany). The concentrations of H_2O_2 and $O^{2\cdot-}$ were determined using a hydrogen peroxide content detection kit (with visible light spectrophotometry, Solarbio, Beijing Solaibao Technology Co., Ltd., Beijing, China, BC3590-50T/48S) and a superoxide anion content test kit (with visible-light spectrophotometry, Solarbio, BC1290-50T/48S), respectively.

2.5. Measurement of Antioxidant Enzyme Activity

The fresh leaves of the seedlings were homogenized in phosphate buffer containing EDTA and polyvinylpolypyrrolidone after being precooled (pH = 7.8) to measure the activities of guaiacol peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD). The activity of ascorbate peroxidase (APX) was estimated by mixing with ascorbic acid, and the supernatant after centrifugation was used as the crude extract for enzyme activity determination.

The activities of SOD, POD, APX and CAT were detected using methods in previous studies with some modifications [5,18–20].

2.6. Analysis of Root Activity in Plants

The root activity was measured using the triphenyl tetrazolium chloride (TTC) method with some modifications [21]. In detail, we soaked 0.2 g fresh root tips (1 cm) in a 10 mL mixture of 0.4% TTC and 0.1 mol·L^{-1} phosphate buffer (pH 7.0), and stored the mixture in darkness at 37 °C for 4 h. After stopping the reaction with 1 mol·L^{-1} H_2SO_4, the root tips were extracted with ethyl acetate. The extract was used to detect the absorbance at 485 nm, and the root activity was calculated using the formula TTC reduction amount (μg)/100 fresh root tips × time (h).

2.7. Analysis of NO^{3-}, Metal ion Concentrations and Nitrate Reductase (NR) Activity in Plants

Fresh tissue from seedlings (l g) was homogenized with deionized water and boiled for 30 min. After filtration, the liquid product was saved and used for analysis. The extracts were mixed with 5% salicylic acid in concentrated H_2SO_4. After cooling down to room temperature, 8% NaOH was added to bring the pH of the mixture to above 12. The absorbance at 410 nm of the mixture was determined as the NO^{3-} content [5,22].

Samples were homogenized in solution mixed with KH_2PO_4, 2% 1-propanol and KNO_3. After that, the mixture was vacuum infiltrated and incubated in a shaking water bath in the dark for 1 h. Sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride were added to the mixture from the previous step. The absorbance at 540 nm of the compound was detected to determine the NR activity [16,17].

2.8. Real-Time Quantitative Analysis

After the extraction and purification of RNA, qRT-PCR was performed following the method of [23] with some modifications. The gene primer sequence of this study was designed using Primer Premier software 6.0, as shown in Table S1, Supplementary Materials. Three separate biological experiments were conducted and repeated three times.

2.9. Analyses of Data and Statistics

SPSS (version 26.0) software was used to perform the statistical analyses. One-way ANOVA 2.0 combined with Duncan's multivariate range test was used to analyze the significant differences among all treatments, and $p < 0.05$ was used as the threshold.

3. Results

3.1. Effect of Exogenous FA on the Inhibited Growth of Seedlings Induced by $Ca(NO_3)_2$ Stress

Superfluous $Ca(NO_3)_2$ had a remarkable adverse effect on the growth of Chinese flowering cabbage (Figure S1). As the treated concentration of $Ca(NO_3)_2$ increased, all the growth indices decreased. $Ca(NO_3)_2$ (80 mM) had an obvious inhibitory effect on plant growth. Compared with the Con, the plant height was obviously reduced by 27.8% with 80 mM $Ca(NO_3)_2$ (Figure S1a). In addition, there were 45.8% and 51% reductions in the fresh weight of shoots and roots, respectively, when using 80 mM $Ca(NO_3)_2$ (Figure S1c,d). The shoot dry weight was reduced by 14.9% with 80 mM $Ca(NO_3)_2$ (Figure S1e). Therefore, 80 mM $Ca(NO_3)_2$ was chosen as the main concentration to conduct the following experiments. After that, we performed a screening test for FA. As shown in Figure 1, the root lengths, compared with 80 mM $Ca(NO_3)_2$, and the fresh and dry weights of the shoots and roots, were increased by 26.67%, 49.2%, 56.47% and 58.31%, respectively, with 100 µM FA treatment. The results revealed that 100 µM FA could significantly alleviate the growth inhibition of Chinese flowering cabbage induced by $Ca(NO_3)_2$ stress.

3.2. Effect of FA Application on the Photosynthetic Capacity of Seedlings under $Ca(NO_3)_2$ Stress

As shown in Figure 2, calcium nitrate stress greatly inhibited photosynthesis in Chinese flowering cabbage seedlings. In detail, compared with the Con, the net photosynthetic rate, transpiration rate and stomatal conductance decreased by 24%, 52% and 67%, respectively, with $Ca(NO_3)_2$ stress (Figure 2a,c,d). However, the application of FA significantly increased the transpiration rate, net photosynthetic rate and stomatal conductance of Chinese flowering cabbage seedlings by 25%, 29% and 36%, respectively. These results revealed that FA had the capacity to alleviate the inhibitory effects on the photosynthetic capacity of seedlings induced by calcium nitrate stress.

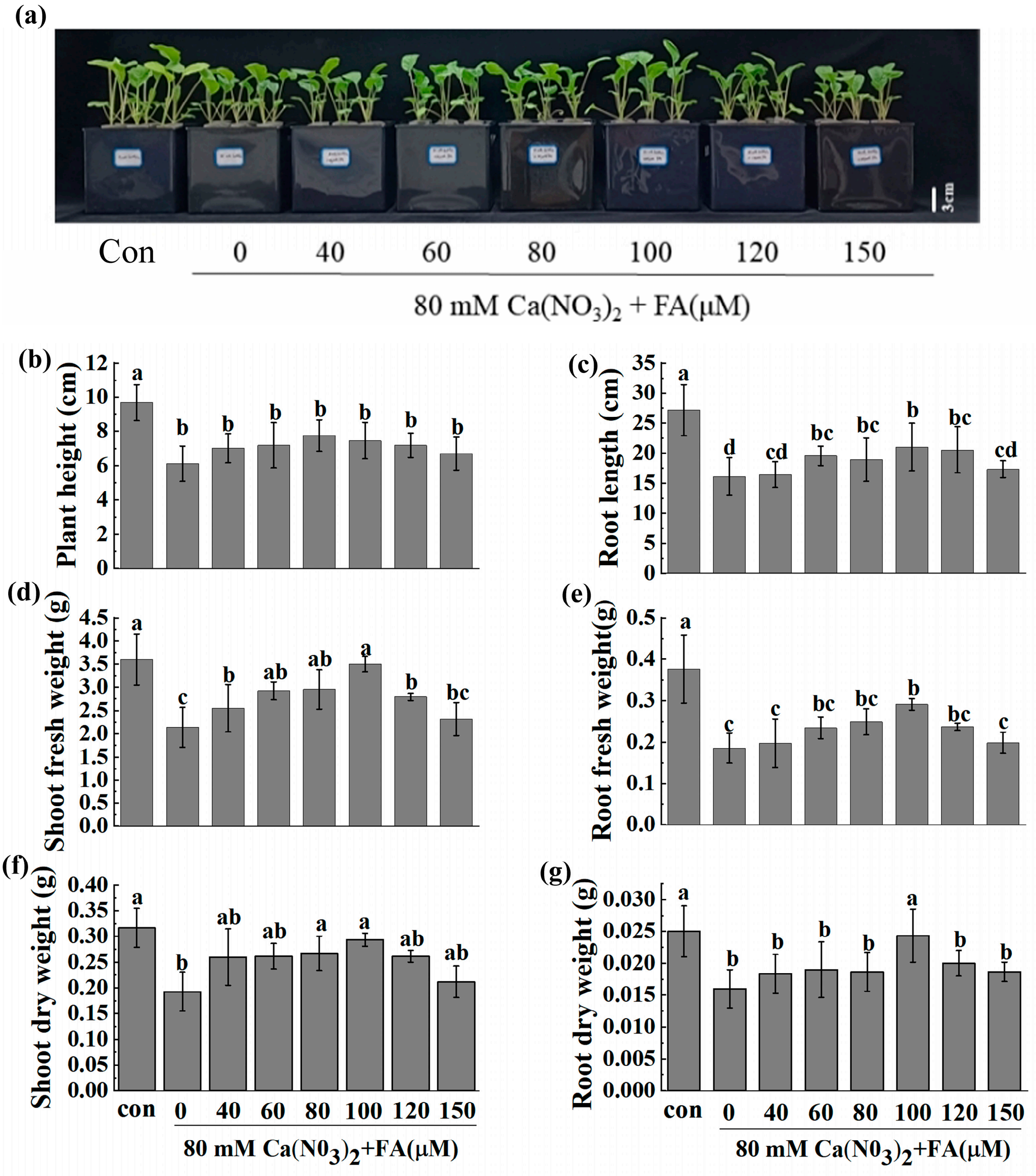

Figure 1. Effect of FA on the growth indices of Chinese flowering cabbage under $Ca(NO_3)_2$ stress: (**a**) stands for the phenotype of Chinese flowering cabbage with different treatments. The plant height (**b**), root length (**c**), shoot fresh weight (**d**), root fresh weight (**e**), shoot dry weight (**f**) and root dry weight (**g**) of seedlings with different concentrations of FA treatment. The plants cultivated in quarter-strength Hoagland's solution with 15 mM $Ca(NO_3)_2$ were used as the control. The treatment groups were 80 mM $Ca(NO_3)_2$ with different concentrations of FA, respectively. After different treatments for 5 d, the growth indices were determined with three independent replicates of 20 plants each. Different letters indicate a significant difference at $p \leq 0.05$.

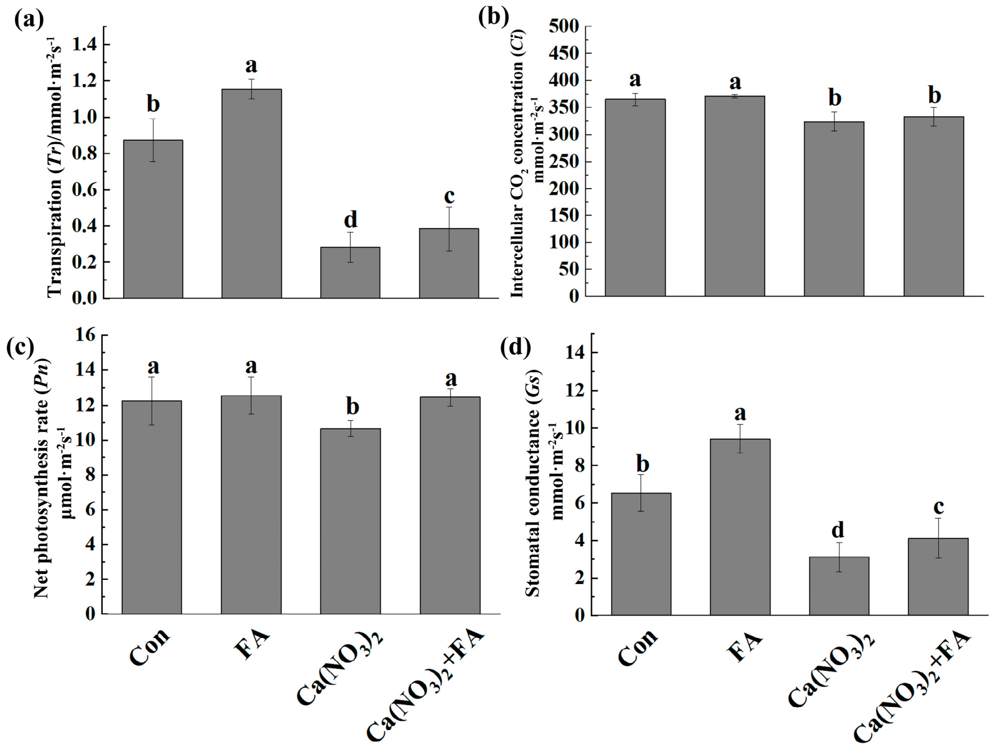

Figure 2. Effects of FA on photosynthetic parameters of Chinese flowering cabbage under $Ca(NO_3)_2$ stress. Measurements of (**a**) transpiration rate (*Tr*), (**b**) intercellular CO_2 concentration (*Ci*), (**c**) net photosynthetic rate (*Pn*) and (**d**) stomatal conductance (*Gs*) with different treatments. The indices were determined with three independent replicates of 10 plants each (n = 10). Different letters indicate a significant difference at $p \leq 0.05$.

3.3. Effect of FA Application on the Oxidation-Reduction System and Root Activity of Seedlings under $Ca(NO_3)_2$ Stress

DAB and NBT histochemical staining were used to evaluate the accumulation of $O^{2\cdot-}$ and H_2O_2 in the leaves of Chinese flowering cabbage. A deeper brown color was observed in the $Ca(NO_3)_2$ treatment, but a lighter brown color was observed in the FA + $Ca(NO_3)_2$ cotreatment (Figure 3a), which indicated that the $O^{2\cdot-}$ accumulation was reduced by FA. Similarly, the H_2O_2 staining experiment showed that darker blue was found in $Ca(NO_3)_2$ treatment alone, but lighter blue was observed in FA + $Ca(NO_3)_2$ cotreatment (Figure 3b), which revealed that the H_2O_2 accumulation was decreased by FA. Compared with the Con, nitrate excess induced the accumulation of $O^{2\cdot-}$, H_2O_2 and MDA, and the relative electrical conductivity 2- or 3.3-fold (Figure 3c–f). However, compared with the $Ca(NO_3)_2$ treated alone, the concentrations of $O^{2\cdot-}$, H_2O_2, MDA and the relative electrical conductivity of plant samples decreased by 30%, 16.7%, 36.9% and 28% with FA application, respectively (Figure 3c–f). This result suggested that FA could alleviate the oxidative damage induced by calcium nitrate stress. As shown in Figure 4, compared with the Con, the activities of SOD, POD, APX and CAT in Chinese flowering cabbage were enhanced to some degree by excess $Ca(NO_3)_2$, which increased by 37.5%, 69.64%, 33.33% and 27.27%, respectively (Figure 4). When treated with 100 μM FA, the reductase activities of SOD, POD, APX and CAT were further increased. That is, compared with $Ca(NO_3)_2$ treatment alone, the activities of SOD, POD, APX and CAT were observably increased by 8.3%, 9%, 18.2% and 6.7% with FA, which revealed that FA was able to strengthen the salt tolerance by improving the antioxidant ability of Chinese flowering cabbage.

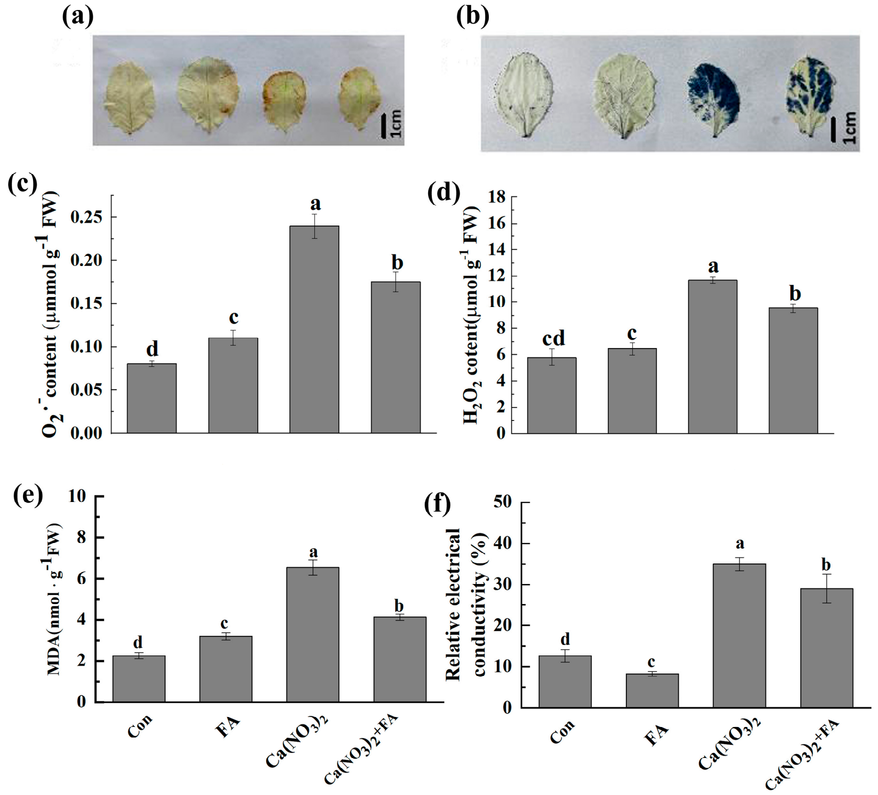

Figure 3. Effect of FA on histochemical localization and concentrations of ROS, MDA and relative electric conductivity in Chinese flowering cabbage under $Ca(NO_3)_2$ stress. The histochemical localization of $O^{2\cdot-}$ (**a**) and H_2O_2 (**b**) in leaves. The accumulation of $O^{2\cdot-}$, (**c**), H_2O_2 (**d**), MDA (**e**) and the relative electric conductivity (**f**) in leaves with different treatments. The plants cultivated in quarter-strength Hoagland's solution with 15 mM $Ca(NO_3)_2$ were the control. Different letters indicated a significant difference at $p \leq 0.05$.

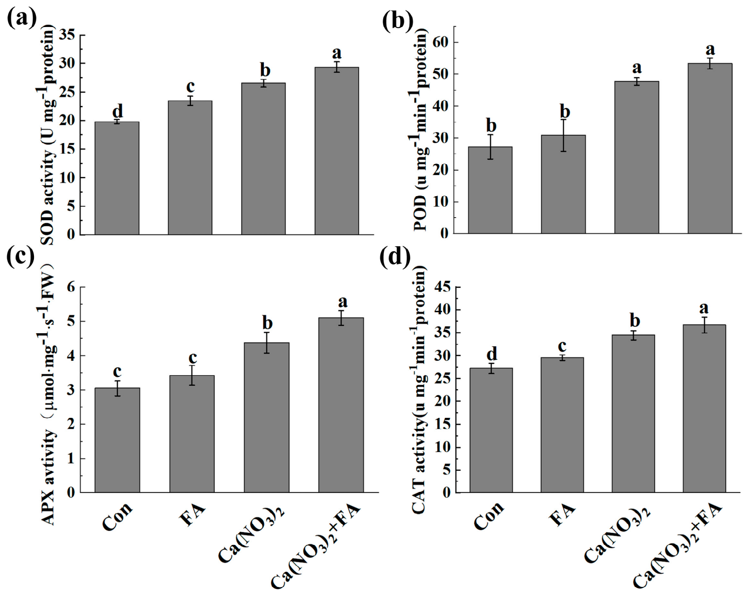

Figure 4. Effect of FA on the activities of SOD (**a**), POD (**b**), APX (**c**) and CAT (**d**) in Chinese flowering cabbage seedlings under $Ca(NO_3)_2$ stress. The plants cultivated in quarter-strength Hoagland's solution with 15 mM $Ca(NO_3)_2$ were the control. Different letters indicate a significant difference at $p \leq 0.05$.

Root activity can directly affect the growth conditions of plants. Compared with Con, $Ca(NO_3)_2$ stress dramatically inhibited the root activity of Chinese flowering cabbage by 34.5%. However, the application of FA alleviated the inhibitory effect of $Ca(NO_3)_2$ stress on root activity. The root activity of Chinese flowering cabbage was increased by 24% with the FA+ $Ca(NO_3)_2$ cotreatment (Figure 5).

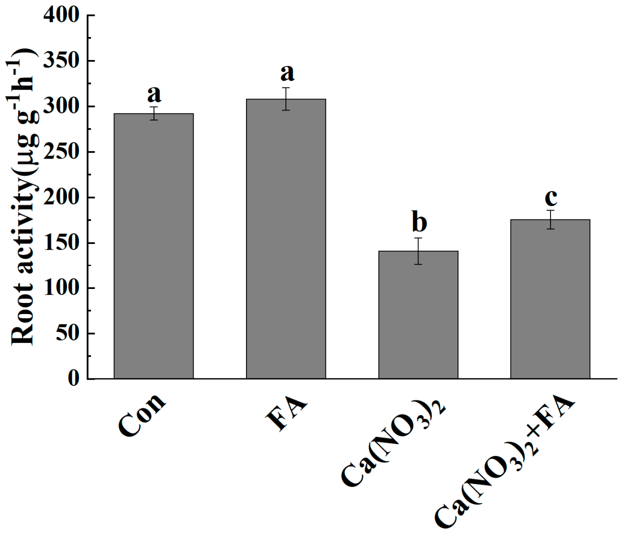

Figure 5. Effect of FA on root activity of Chinese flowering cabbage seedlings under $Ca(NO_3)_2$ stress. The plants grown cultivated in quarter-strength Hoagland's solution with 15 mM $Ca(NO_3)_2$ were the control. Different letters indicate a significant difference at $p \leq 0.05$.

3.4. Effect of Exogenous FA on Nitrate Content, NO^{3-}-N Ratio (Shoot: Root) and Nitrate-Related Enzyme Activity in Seedlings under $Ca(NO_3)_2$ Stress

The nitrate content and ratio of NO^{3-}-N (shoot/root) of Chinese flowering cabbage were determined (Figure 6). Compared with the Con, the nitrate contents of the shoots and roots were significantly increased by 55% and 105% with $Ca(NO_3)_2$ alone (Figure 6a). When cotreated with FA, the nitrate concentrations of roots and shoots were reduced by 27.7% and 12%, respectively (Figure 6a). In addition, $Ca(NO_3)_2$ + FA treatment could decrease the shoot–root translocation ratio of $Ca(NO_3)_2$, which could retain more $Ca(NO_3)_2$ in the root and protect the shoot growth of seedlings from nitrate toxicity (Figure 6b). It has been reported that the NRT1 gene family is involved in the absorption and transfer of nitrate [5]. Similarly, the transcriptional levels of *BcNRT1.1* and *BcNRT1.5* were studied. The expression of *BcNRT1.1* and *BcNRT1.5* was significantly induced by $Ca(NO_3)_2$ (Figure 6c,d). However, FA observably suppressed the transcriptional levels of *BcNRT1.1* and *BcNRT1.5* (Figure 6c,d), which suggested that the $Ca(NO_3)_2$ absorption and transport capacities were decreased by FA. The above findings revealed that FA reduced $Ca(NO_3)_2$ absorption and translocation by depressing the transcriptional levels of *BcNRT1.1* and *BcNRT1.5*, which finally reduced the concentration of $Ca(NO_3)_2$ in plants.

As shown in Figure S2, NR activity was significantly induced by $Ca(NO_3)_2$ alone compared to the Con. In addition, $Ca(NO_3)_2$ + FA cotreatment further improved NR activities in roots by 15%. The activity of NR in the shoots was induced by FA to some extent without significance. This result suggested that FA can decrease $Ca(NO_3)_2$ accumulation in seedlings by influencing NO^{3-} metabolism.

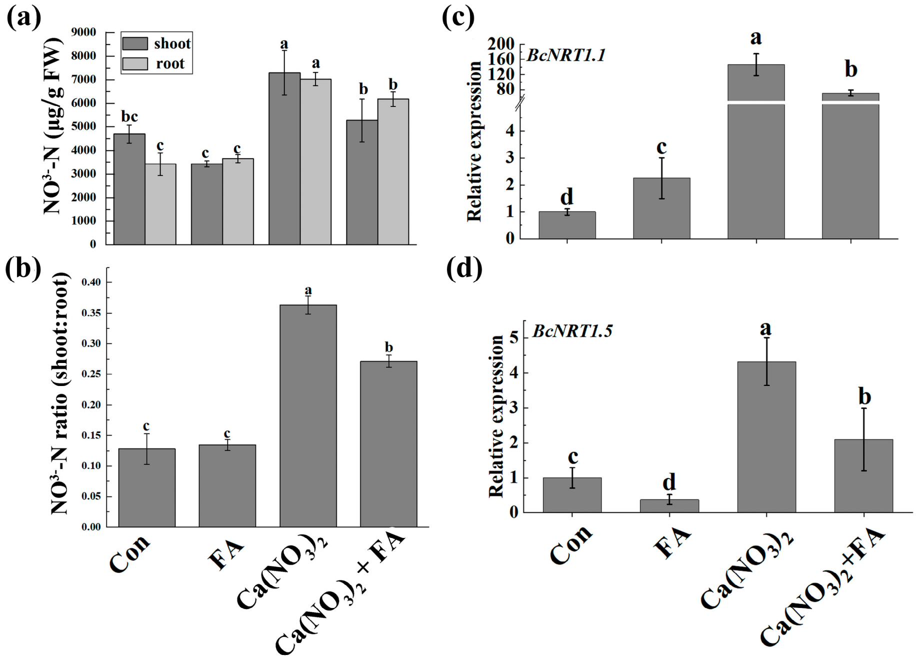

Figure 6. Effect of FA on NO^{3-} content, NO^{3-}-N ratio (shoot/root) and nitrate relative genes in Chinese flowering cabbage seedlings under nitrate stress: (**a**) represents the content of NO^{3-}; (**b**) shows the NO^{3-}-N ratio (shoot/root) in different treatments; (**c**,**d**) represent the relative expression of *BcNRT1.1* and *BcNRT1.5*. The plants grown in quarter-strength Hoagland's solution with 15 mM $Ca(NO_3)_2$ were the control. Different letters indicate a significant difference at $p \leq 0.05$.

4. Discussion

Soil secondary salinization is one of the main causes of continuous obstacles to vegetable cropping. Soil secondary salinization may result from the accumulation of soil nitrate nitrogen caused by excess nitrogen fertilizer application [5]. Excessive nitrate can lead to serious adverse effects on vegetable growth and nutrient quality. At present, many exogenous substances and chemical fertilizers, including hydrogen-rich water (HRW), spermidine (Spd) and melatonin (MT), have been used to alleviate nitrate stress [5,24–26]. However, the alleviatory effect of exogenous FA on $Ca(NO_3)_2$ stress in Chinese flowering cabbage has not been analyzed. In this study, 100 μM FA alleviated the inhibitory effect of 80 mM $Ca(NO_3)_2$ on the growth of Chinese flowering cabbage (Figure 1). As shown in Figure 7, the alleviation of nitrate stress by FA may result from the positive effect on the photosystem and oxidation-reduction system, but it decreased the accumulation of nitrate in Chinese flowering cabbage seedlings (Figure 7).

We found that the salt stress caused by $Ca(NO_3)_2$ could observably reduce photosynthesis in Chinese flowering cabbage seedlings (Figure 2), which was in accord with the inhibitory effect of nitrate stress on tomato and pak choi seedlings [2,5]. FA could significantly improve the photosynthetic capacity of Chinese flowering cabbage seedlings under nitrate stress by increasing the transpiration rate, stomatal conductance and net photosynthetic rate. Likewise, the weakened photosynthetic capacity induced by salt stress in tomato can also be alleviated by changing these photosynthetic parameters with far-red light treatment [25]. Many studies have found that $Ca(NO_3)_2$ can greatly induce $O^{2\cdot-}$, H_2O_2 and MDA accumulation, and the activities of antioxidant enzymes are adaptively enhanced to resist nitrate stress in pakchoi and tomato seedlings [5,26]. In our study, the application of FA significantly reduced ROS levels and membrane lipid peroxidation, as determined via histochemical localization and concentration analyses (Figure 3). In addition, plants can produce enzymatic scavengers, including SOD, POD, CAT and APX, to protect seedlings from oxidative stress. SOD is involved in clearing the ROS, and POD, APX and CAT can remove H_2O_2. Antioxidase activity could be further increased to fight free radical damage [27]. Our results suggested that excess $Ca(NO_3)_2$ increased antioxidant enzyme activity, which was further improved by FA (Figure 4). This result was consistent with the report by [5,26,27].

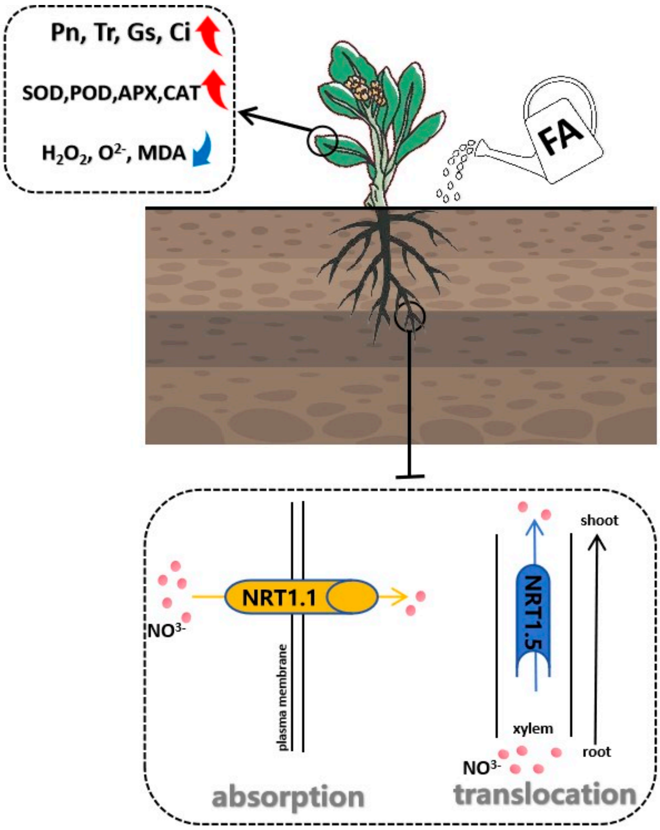

Figure 7. The mechanistic model of Chinese flowering cabbage in the alleviation of nitrate stress by FA. The growth depression of Chinese flowering cabbage induced by $Ca(NO_3)_2$ was alleviated by FA. FA could promote photosynthetic capacity by increasing Pn, Tr, Gs and Ci. In addition, the activity of reductases including SOD, POD, APX and CAT was increased, but the accumulations of H_2O_2, O^2 and MDA were decreased by FA under $Ca(NO_3)_2$ stress. Moreover, FA inhibited the expressions of *BcNRT1.1* and *BcNRT1.5*, which suppressed the uptake and translocation of $Ca(NO_3)_2$. In conclusion, the possible reasons for the alleviation of nitrate stress by FA in Chinese flowering cabbage seedlings were explored in this study through analyses of the photosystem, oxidation-reduction system and nitrate accumulation.

Many studies have found that FA has great potential in improving stress resistance, increasing crop yield and maintaining sustainable agricultural development [5,28–30]. However, the application of FA to Chinese flowering cabbage under nitrate stress has rarely been reported. In this study, 100 μM FA significantly alleviated the growth inhibition of Chinese flowering cabbage caused by $Ca(NO_3)_2$ stress. In addition, the analysis results of the photosynthetic system showed that the net photosynthetic rate, transpiration rate and stomatal conductance under nitrate stress were improved by the application of FA (Figure 2). This result suggested that FA had the capacity to improve the abnormal photosystem induced by nitrate stress. Beyond that, some studies have shown that the application of FA could enhance enzyme activity and increase crop yield [5,28,29,31]. Our analysis results of the oxidation-reduction system also revealed that FA decreased the accumulation of $O^{2\cdot-}$, H_2O_2 and MDA induced by $Ca(NO_3)_2$ stress, but enhanced the activities of SOD, POD, APX and CAT in Chinese flowering cabbage, which finally relieved the oxidative stress induced by calcium nitrate. All the results suggested that FA can alleviate $Ca(NO_3)_2$ stress in Chinese cabbage seedlings by improving the antioxidative capacity against oxidative damage.

NRT1.1 is involved in nitrate absorption, and NRT1.5 and NRT1.8 are identified as two transporters for root-to-shoot nitrate translocation [32–34]. In the nitrate content experiment, we observed that FA could reduce nitrate accumulation in roots (Figure 6a), which was in accord with the transcription level of *BcNRT1.1* (Figure 6c).Meanwhile, FA could also decrease nitrate translocation (Figure 6b), which was in accord with the gene expression of *BcNRT1.5* (Figure 6d). *NRT1.8* could not be detected in Chinese flowering cabbage seedlings in our study. Overall, we speculated that FA-mediated amelioration of nitrate stress may result from its inhibitory effect on nitrate absorption and translocation in Chinese cabbage flowering seedlings.

5. Conclusions

FA (100 μM) improved the growth of Chinese flowering cabbage seedlings under $Ca(NO_3)_2$ stress. The degree of oxidative damage induced by $Ca(NO_3)_2$ stress could be alleviated by FA, which resulted from both the reduced concentrations of $O^{2\cdot-}$, H_2O_2 and MDA and the increased activities of antioxidant enzymes resulting from FA application. Moreover, FA effectively reduced the accumulation of nitrate by decreasing the absorption and shoot–root translocation of nitrate by regulating the transcriptional levels of *BcNRT1.1* and *BcNRT1.5*. Taken together, exogenous FA may alleviate nitrate stress by improving oxidation resistance, photosynthetic capacity and redundant $Ca(NO_3)_2$ accumulation in Chinese flowering cabbage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app132212373/s1. Table S1: Primers for qRT-PCR; Figure S1: Effect of $Ca(NO_3)_2$ on the growth indices of Chinese flowering cabbage seedlings. Figure S2: Effect of FA on NR activity in Chinese flowering cabbage seedlings under nitrate stress.

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