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Thymol Deploys Multiple Antioxidative Systems to Suppress ROS Accumulation in Chinese Cabbage Seedlings under Saline Stress

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Abstract: Developing biostimulants is a promising approach for sustainable agriculture under a saline environment. Thymol is a plant-derived compound with a potential antioxidative capacity. However, little is known about whether and how the antioxidative property of thymol plays a role in inducing plant tolerance against abiotic stresses. Here, we find that thymol induces saline tolerance in Chinese cabbage seedlings via enhancing the antioxidative capacity. Treatment with NaCl (100 mM) decreased the seedling fresh weight by 59.9% as compared to a control. Thymol at 20 μM showed the greatest effect on promoting seedling growth under saline stress, with the seedling fresh weight being increased by 71.0% as compared to NaCl treatment. Thymol remarkably decreased the overaccumulation of ROS (hydrogen peroxide and a superoxide radical); cell membrane damage (evaluated by lipid oxidation, membrane integrity, and relative conductivity); and cell death in seedlings under saline stress. Thymol induced three antioxidative systems to lower the ROS level in salt-treated seedlings. First, thymol remarkably activated a set of antioxidative enzymes, such as SOD (superoxide dismutase), APX (ascorbate peroxidase), CAT (catalase), and POD (peroxidase). Second, thymol balanced the cellular redox status by increasing the ratio of AsA/DHA (ascorbic acid/dehydroascorbic acid) and GSH/GSSG (glutathione/oxidized glutathione). Third, thymol significantly enhanced the level-two kinds of antioxidants (total phenol and flavonoid). All of these physiological responses were observed in both the shoots and the roots. In sum, thymol deploys multiple antioxidative systems to help Chinese cabbage seedlings against saline stress. Such findings suggest that thymol has great potential to be developed as a novel biostimulant enhancing crop tolerance against saline stress.

Keywords: antioxidant; biostimulant; Chinese cabbage; oxidative stress; ROS; salinity; thymol

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1. Introduction

Variable environmental challenges drive us to seek approaches maintaining crop production. Developing novel biostimulants to help crops combat environmental stimuli has been drawing great attention in resilient agriculture [1]. Biostimulants can trigger crop tolerance, facilitating crop growth and production under stressful conditions. Biostimulants can be developed based on both synthetically created chemicals or natural resources (such as substances and metabolites from plants or microbes). Compared to the uncertain toxicity and environmental adaptability of synthetic chemicals, natural chemicals may have more applicational potential in agriculture. Plants are natural stock with various chemicals,

providing important resources for the development of plant-based natural biostimulants for sustainable agriculture [2].

Thymol is a kind of natural phenol, the main component of essential oils extracted from the *Thymus* species. It also exists in other plant species at a low content. Thymol has been selected for therapeutic applications, as most of the *Thymus* species are medicinal herbs [3]. Besides medicinal practice, thymol has great potential to be applied in agriculture. The practical application of thymol as an agrochemical has been promising, but most of the reports focus on the antimicrobial activity of thymol against plant pathogens [4]. Whether thymol can be used as a biostimulant remains elusive. Our previous studies found the bioactivity of thymol in inducing plant tolerance against abiotic stress (e.g., heavy metals, ammonium, and salinity). In these studies, thymol helped to suppress the accumulation of ROS (reactive oxygen species) in plants under stressful conditions [5–7]. However, how thymol modulates ROS homeostasis in plants to combat abiotic stress is not fully understood.

Soil salinity is getting worse in agricultural environments due to climate change and anthropogenic activities [8]. Saline stress dampens seed germination, crop growth, and production yields [9]. The over-accumulation of reactive oxygen species (ROS) results in cell death via membrane lipid peroxidation in plant cells upon saline stress. Maintaining ROS homeostasis is important for plant salinity acclimation [10]. The superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are two representatives of free radical and non-radical forms of ROS, respectively [11]. Plants have several systems to eliminate excessive ROS induced by saline stress. The first system consists of antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate oxidase (APX) [12,13]. The second system consists of several antioxidants, such as phenol, flavonoid, glutathione (GSH), and ascorbic acid (AsA), etc. [14,15]. In addition, the redox balance controlled by the reduced status of GSH and AsA plays a role in antagonizing oxidized plant cells caused by ROS accumulation under saline stress [16].

Plant saline tolerance can be regulated exogenously [17,18]. This promotes the rapid development of biostimulants to improve crop saline tolerance [19]. These biostimulants facilitate saline tolerance via the regulation of plant intrinsic physiology, including the redox balance, photosynthesis reinforcement, osmotic adjustments, and carbohydrate metabolism, etc. [20–22] The salt-resistant function of biostimulants has been identified, but the potentiality of applying them in agriculture remains elusive due to limited environmental adaptations. Therefore, developing novel biostimulants that can be used in agriculture is still needed. Thymol has been applied as an effective fungicide, showing a great potential for applications [23].

The pharmacological property of thymol has been associated to the activation of antioxidative enzymes (e.g., SOD, POD, and CAT) and the enhancement of vitamin C and vitamin E in mammals [24]. Whether thymol regulates plant saline tolerance through antioxidative systems remains unclear. In this study, we determined the capability of thymol in regulating saline tolerance in Chinese cabbage seedlings by evaluating the seedling growth, oxidative injuries, and multiple antioxidative systems. The results will extend the biological function of thymol and will elucidate the physiological mechanism for thymol-induced saline tolerance in plants. This would help us understand the potential of using thymol as a biostimulant in agriculture based on its antioxidative properties.

2. Materials and Methods

2.1. Seedling Growth and Treatment

The seeds of Chinese cabbage (*Brassica rapa*, Shanghai Qing) were obtained from Nanjing Luling Seed Industry Co., Ltd. (Nanjing, China). The seeds were sterilized with 0.5% NaClO for 5 min, followed by washing with distilled water to remove NaClO residues on the seed surface. Then, the seeds were placed on saturated filter papers to germinate in darkness for 24 h. Germinated seeds were allowed to grow radicles in an incubator at a temperature of 25 °C, a photoperiod of 16/8 h, and a photosynthetic radiation of

250 μmol/m^2/s. Seedlings with a 1 cm root were transferred to a Petri dish with three filter papers saturated with Hoagland nutrition (1/4 strength). NaCl (25, 50, 100, 200 mM) or thymol (5, 10, 20, 40) was mixed with the Hoagland nutrition to treat the seedlings. The seedlings grew in the same incubator as mentioned above. For the time-course experiments, the seedlings were treated for 6, 12, 24, 48, and 72 h, respectively. For other experiments, the seedlings were treated for 72 h. After treatment, the roots and shoots were harvested, respectively, for physiological measurement.

2.2. Seedling Growth Measurements

The root length was measured with a ruler (10 replicates for each treatment). The seedlings, after treatment, were surface-dried gently with filler papers before weighing. For each replicate, 10 seedlings were weighed together in order to obtain an accurate fresh weight. The shoots and roots were weighed, respectively, with 3 replicates for each treatment.

2.3. Histochemical Staining

Two ROS, H_2O_2 and $O_2^{\bullet-}$, in the leaves were evaluated histochemically in vivo. DAB (3,3-diaminobenzidine) was used to detect endogenous H_2O_2 in the leaves. The leaves of the seedlings were stained with 0.1% (*w*/*v*) of a DAB solution for 30 min, allowing for the reaction between H_2O_2 and DAB to generate brown products. Then, the stained leaves were boiled in 95% ethanol (*v*/*v*) to remove chlorophyll in order to photograph the brown products clearly [25]. NBT (nitro-blue tetrazolium) was used to detect endogenous $O_2^{\bullet-}$ in the leaves. The leaves of seedlings were stained with 10 mM of an NBT solution for 30 min, allowing for the reaction between $O_2^{\bullet-}$ and NBT to generate dark-blue products. Then, the chlorophylls were removed, as mentioned above, before photographing [25].

Dead cells in the seedlings were stained with trypan blue [26]. The roots were stained with trypan blue (15 mg/mL) for 30 min, followed by washing with distilled water and photographing. The leaves were stained with trypan blue for 5 h, followed by removing chlorophylls and photographing, as mentioned above.

Evans blue was applied to indicate the damage of the plasma membrane integrity [27]. The roots were stained with 0.025% (*w*/*v*) Evans blue for 20 min, followed by washing with distilled water and photographing. The leaves were stained with Evans blue for 5 h, followed by removing chlorophylls and photographing, as mentioned above.

2.4. Fluorescent Detection In Vivo

Several oxidation-related indicators in roots were evaluated in vivo with specific fluorescent probes. The total ROS in the roots were labeled with probe DCFH-DA (2′,7′-dichlorofluorescein diacetate). The roots were incubated in DCFH-DA (5 μM) for 20 min to allow the probe to enter into the root cells. Then, the intracellular ROS labeled with DCF emitted a green fluorescence that could be observed and photographed under a fluorescent microscope (ECLIPSE, TE2000-S, Nikon, Melville, LA, USA).

The H_2O_2 in the roots were labeled with probe HPF (3′-(*p*-hydroxyphenyl) fluorescein) [5]. The roots were incubated in HPF (5 μM) for 20 min, emitting a green fluorescence under the fluorescent microscope. The $O_2^{\bullet-}$ in the roots were labeled with probe DHE (dihydroethidium) [28]. The roots were incubated in a DHE solution (15 μM) for 20 min, emitting a red fluorescence under the fluorescent microscope.

Lipid peroxidation in the root cells was indicated with probe C11-BODIPY [29]. The roots were incubated in a C11-BODIPY solution (10 μM) for 20 min, emitting a red fluorescence under the fluorescent microscope.

Cell death in the roots was also indicated with probe PI (propidium iodide) [30]. The roots were incubated in a 10 μM PI solution for 25 min. Dead cells labeled with PI emitted a red fluorescence under the fluorescent microscope.

2.5. Determination of Relative Conductivity

The roots and shoots of the seedlings after treatment were harvested, respectively, for the determination of relative conductivity. Plant samples were washed gently with distilled water, followed by incubating in distilled water at 25 °C for 3 h. Then, the conductivity of the incubation solution was determined as C_1. Then, these plant samples were incubated in distilled water at 100 °C for 1 h, allowing the temperature to drop to 25 °C. The conductivity of this solution was determined as C_2. The conductivity of the distilled water (25 °C) without any samples was determined as C_0. The relative conductivity of the plant samples was quantified as $(C_1 - C_0)/(C_2 - C_0)$.

2.6. Assay of Enzyme Activity

Fresh plant samples were homogenized with a precooled phosphate buffer solution (pH 6.8, 50 mM) containing 1% *w*/*v* polyvinylpyrrolidone. For the determination of APX, AsA (50 μM) was also added to the extraction buffer in order to maintain the stability of APX. The homogenate was centrifuged for 30 min (12,000× *g*, 4 °C). The supernatant was harvested for the assay of enzyme activity by using several commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activity of SOD was measured using the kit A001-1-2 based on quantifying the inhibition of the reaction between superoxide radicals and hydroxylamine, yielding nitrite that reacted with 4-aminobenzenesulfonic acid and methyl naphthylamine to form products, with absorbance at 550 nm. The activity of APX was measured with the kit A123-1-1 based on the oxidation rate of AsA by H_2O_2 ($OD_{290\ nm}$). The activity of CAT was measured with the kit A007-1-1 based on quantifying the inhibition of CAT-mediated H_2O_2 decomposition by adding ammonium molybdate that reacts with H_2O_2 to yield products with absorbance at 405 nm. The activity of POD was measure with the kit A084-3-1 based on the oxidation rate of tetramethyl benzidine in the presence of H_2O_2 ($OD_{420\ nm}$) [31].

2.7. Evaluation of Celllular Redox Status

The cellular redox status was evaluated based on the ratio of AsA/DHA (dehydroascorbic acid) and GSH/GSSG (oxidized glutathione). The fresh seedling samples were homogenized with a cooled phosphate buffer solution (pH 7.4, 100 mM), followed by centrifuging for 20 min (10,000× *g*, 4 °C). The supernatant was collected for measuring the content of metabolites. The content of AsA, GSH, and GSSG were determined by using several commercial kits obtained from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. The AsA content was measured with the kit A009-1-1 based on the reduction of Fe^{3+} by AsA to generate Fe^{2+}, which further reacts with phenanthroline to yield products with absorbance at 536 nm. The GSH content was measured with the kit A006-2-1 based on the reaction of GSH and dithiodinitrobenzoic acid (DTNB), yielding products with absorbance at 405 nm. The GSSG content was measured with the kit A061-1-2. Glutathione reductase was added to transform all the GSSG to GSH. Then, the GSH content was measured with the DTNB method in sample extractions before and after adding GR, respectively. The GSSG content was calculated from the difference between two measurements [31,32]. The DHA content was determined by using the commercial kit BC1240 (Solarbio Life Science, Beijing, China). The total AsA was measured after a reduction of DHA to ASA with 1,4-dithiothreitol (DTT), based on the ascorbate oxidase method. The concentration of DHA was calculated from the difference between the total AsA and ASA [33]. Then, the ratio of AsA/DHA and GSH/GSSG was calculated.

2.8. Determination of Metabolites

Plant samples were homogenized and extracted with 60% ethanol. The total phenol content was measured using the commercial kit A143-1-1 (Nanjing Jiancheng Bioengineering Institute) based on the reaction of phenol and tungstic molybdic acid, yielding products with absorbance at 760 nm. The total flavonoid was measured using the commercial kit

A142-1-1 Nanjing Jiancheng Bioengineering Institute), based on the reaction of flavonoids and Al^{3+} in the presence of nitrite, yielding products with absorbance at 502 nm [34,35].

2.9. Determination of Total Soluble Protein Content

The plant samples were homogenized with the methods mentioned in Section 2.7 to extract the total soluble protein. The protein content was measured by using the commercial kit A045-4, based on the Coomassie brilliant blue method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) [31].

2.10. Data Analysis

The results are presented as the mean of 3–10 replicates with SD (standard deviation). The significant difference among the treatments ($p < 0.05$) was evaluated by using the LSD (least significant difference) test with ANOVA (one-way analysis of variance). The package "corrplot (Version 0.92)" was applied to perform a Pearson correlation analysis [36]. For a cluster analysis, the data for each treatment was transformed to Log_2 (fold change) as compared to the control group. Then, TBtools (Version II) was applied to generate heatmaps for the cluster analysis [37].

3. Results

3.1. Salinity Inhibited the Growth of Chinese Cabbage Seedlings

The growth of the Chinese cabbage seedlings was inhibited by the NaCl treatment (Figure 1A). The root length and fresh weight of the seedlings were quantified to evaluate the effect of salinity on seedling growth (NaCl exposure for 72 h). Compared to the control, NaCl at 25, 50, 100, and 200 mM reduced the root length by 28.8%, 37.0%, 42.0%, and 67.5%, respectively (Figure 1B). The time-course experiment indicated that 25 mM of NaCl began to inhibit the root length post exposure of 12 h, while NaCl at 50–100 mM worked at 6 h (Figure 1C). The seedlings' fresh weight decreased upon the increase in NaCl concentration. Compared to the control, the seedlings' fresh weight was decreased by 20.0%, 45.0%, 59.9%, and 71.3% with an NaCl concentration of 25, 50, 100, and 200 mM, respectively (Figure 1D). These results suggest that saline stress significantly inhibited the growth of the Chinese cabbages seedlings in a dose-dependent manner. And 100 mM of NaCl was selected for the following experiments, as it caused moderate growth inhibition.

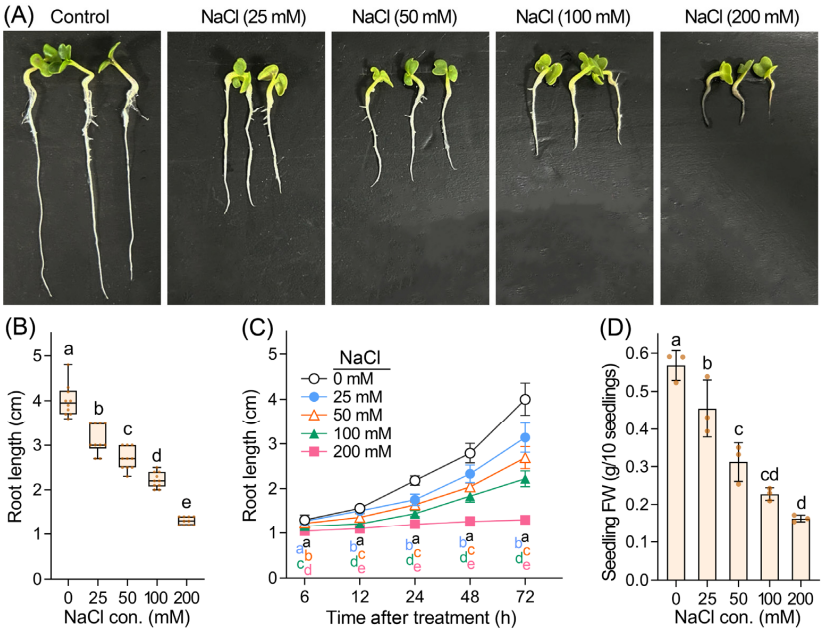

Figure 1. Effects of salinity on the growth of Chinese cabbage seedlings. (**A**) Growth phenotype of seedlings upon exposure of NaCl at different concentrations for 72 h. (**B**) The dose-dependent effects

of NaCl on root length (n = 10). (**C**) The time-dependent effects of NaCl on root length (n = 10). (**D**) The dose-dependent effects of NaCl on seedling fresh weight (n = 3). Different lowercase letters in (**B**–**D**) indicate significant differences among different treatments (ANOVA, $p < 0.05$).

3.2. Thymol Promoted the Growth of Chinese Cabbage Seedlings upon Saline Stress

Thymol at 5–40 μM was applied to evaluate seedling growth under NaCl exposure for 72 h. Compared to the NaCl treatment, the root length increased with the increase in thymol concentration, with a maximum effect occurring at 20 μM (Figure 2A,B). Thymol at 20 μM began to rescue the root growth at 6 h, with an enhanced growth speed as compared to the NaCl treatment (Figure 2C). The fresh weight of the whole seedling, root, and shoot under the NaCl + thymol treatment significantly increased by 56.0%, 19.5%, and 71.0%, respectively, compared to the NaCl treatment (Figure 2D–F). These results suggest that thymol had the capability of rescuing the growth of the Chinese cabbage seedlings under saline stress. Then, we used thymol at 20 μM to study the physiological responses in the seedlings.

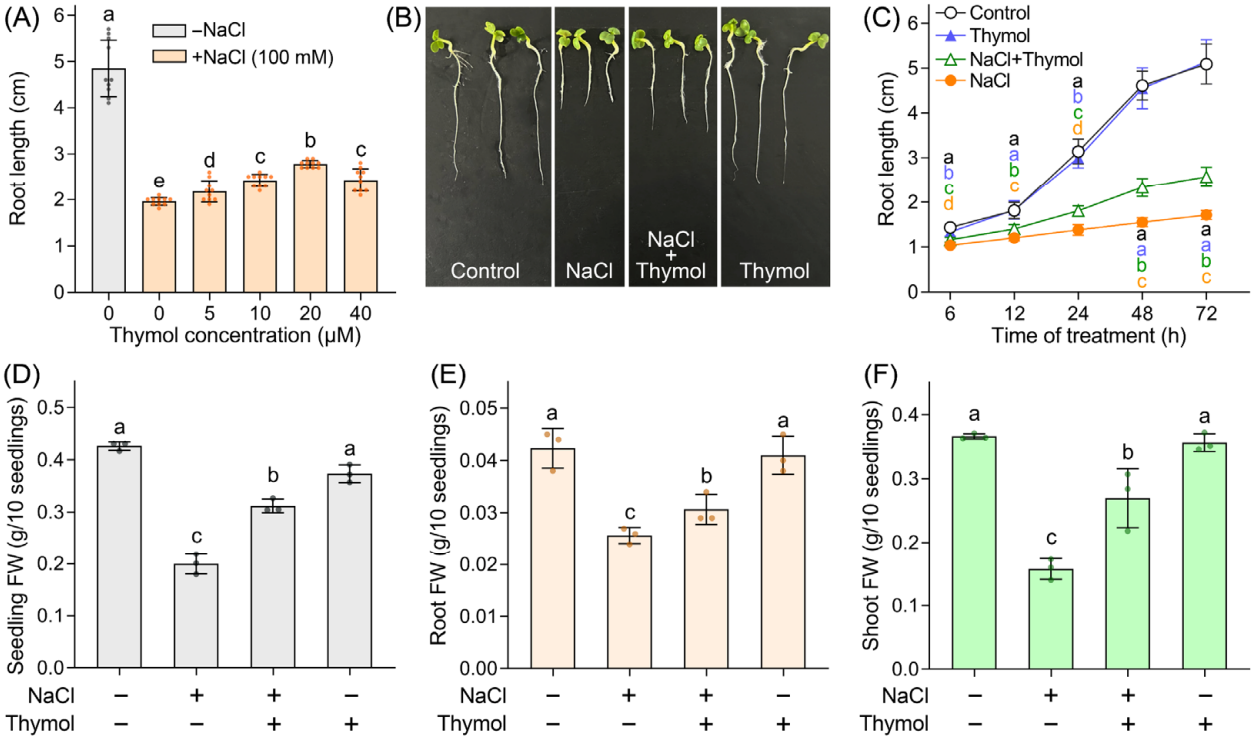

Figure 2. Effects of thymol on the growth of Chinese cabbage seedlings under saline stress. The seedlings were exposed to NaCl or thymol for 72 h. (**A**) Effects of thymol at 5–40 μM on seedling root length under NaCl (100 mM) stress (n = 10). (**B**) Growth phenotype of seedlings upon thymol (20 μM) and NaCl (100 mM) exposure. (**C**) The time-dependent effects of thymol (20 μM) root length under NaCl (100 mM) exposure (n = 10). (**D**) Effects of thymol at 20 μM on seedling fresh weight under NaCl (100 mM) stress (n = 3). (**E**) Effects of thymol at 20 μM on root fresh weight under NaCl (100 mM) stress (n = 3). (**F**) Effects of thymol at 20 μM on shoot fresh weight under NaCl (100 mM) stress (n = 3). Different lowercase letters in (**A**,**C**,**D**–**F**) indicate significant differences among different treatments (ANOVA, $p < 0.05$).

3.3. Thymol-Suppressed ROS Accumulation in Chinese Cabbage Seedlings upon Saline Stress

The ROS levels in the roots were labeled with several kinds of fluorescent probes. NaCl treatment for 72 h caused an accumulation of the total ROS (labeled with DCF fluorescence) in the roots, which was decreased by applying thymol (Figure 3A). The DCF fluorescent density in the thymol + NaCl treatment remarkably decreased by 10.4% as

compared to the NaCl treatment (Figure 3B). Thymol also suppressed the salt-induced accumulation of two typical ROS, H_2O_2 and $O_2^{\bullet-}$, in roots (Supplementary Materials). The HPF fluorescent density (indicating endogenous H_2O_2) in the thymol + NaCl treatment significantly decreased by 11.3% as compared to the NaCl treatment (Figure 3C,D). The DHE fluorescent density (indicating endogenous $O_2^{\bullet-}$) under the thymol + NaCl treatment significantly decreased by 28.2% as compared to the NaCl treatment (Figure 3E,F). Then, we calculated the relative change in $H_2O_2/O_2^{\bullet-}$ in the roots based on the fluorescent of HPF and DHE. Saline stress significantly decreased the $H_2O_2/O_2^{\bullet-}$, which was reversed by adding thymol. Thymol alone failed to change the $H_2O_2/O_2^{\bullet-}$ in the roots (Figure S1). Histochemical staining was used to evaluate the level of endogenous H_2O_2 and $O_2^{\bullet-}$ in the leaves. Thymol decreased the accumulation of H_2O_2 (stained with DAB) and $O_2^{\bullet-}$ (stained with NBT) in the leaves upon saline stress (Figure 3G,H). These results suggest that thymol was able to inhibit salt-induced ROS accumulation in both the roots and the leaves.

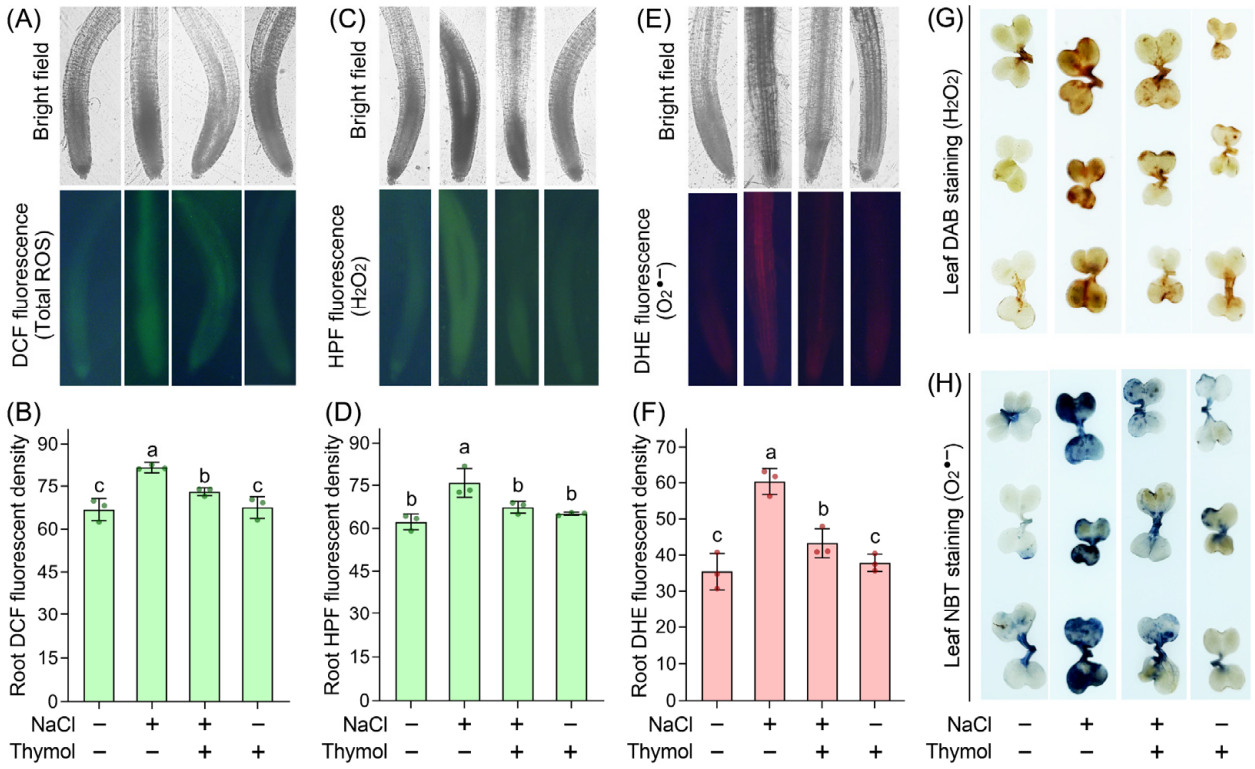

Figure 3. Effects of thymol on ROS accumulation in Chinese cabbage seedlings under saline stress. The seedlings were exposed to NaCl or thymol for 72 h. (**A**) Total ROS level as indicated by DCF fluorescence in roots. (**B**) Relative DCF fluorescent density in roots ($n = 3$). (**C**) H_2O_2 level as indicated by HPF fluorescence in roots. (**D**) Relative HPF fluorescent density in roots ($n = 3$). (**E**) $O_2^{\bullet-}$ level as indicated by DHE fluorescence in roots. (**F**) Relative DHE fluorescent density in roots ($n = 3$). (**G**) H_2O_2 level as indicated by DAB staining in leaves. (**H**) $O_2^{\bullet-}$ level as indicated by NBT staining in leaves. Different lowercase letters in (**B**,**D**,**F**) indicate significant differences among different treatments (ANOVA, $p < 0.05$).

3.4. Thymol Attenuated Saline-Induced Oxidative Damage in Chinese Cabbage Seedlings

The treatment with NaCl for 72 h induced root lipid peroxidation, as indicated by C11 BODIPY fluorescence, which was decreased by thymol (Figure 4A). The corresponding fluorescent density under the thymol + NaCl treatment significantly decreased by 34.1% as compared to the NaCl treatment (Figure 4B). Thymol attenuated the salt-induced plasma membrane damage (indicated by Evans blue staining) in both the leaves and the roots (Figure 4C). The relative conductivity in the shoots and roots under the thymol + NaCl treatment significantly decreased by 36.1% and 22.6%, respectively, as compared to the

NaCl treatment (Figure 4D,E). Thymol attenuated salt-induced cell death in the roots (as indicated with PI fluorescence) (Figure 4F). The PI fluorescence under the thymol + NaCl treatment significantly decreased by 23.1% as compared to the NaCl treatment (Figure 4G). Trypan blue staining also indicated attenuated cell death in the roots and shoots under the thymol + NaCl treatment as compared to the NaCl treatment (Figure 4H). These results suggest that thymol maintained cell membrane integrity and ameliorated oxidative injuries and cell death in the Chinese cabbage seedlings under saline stress.

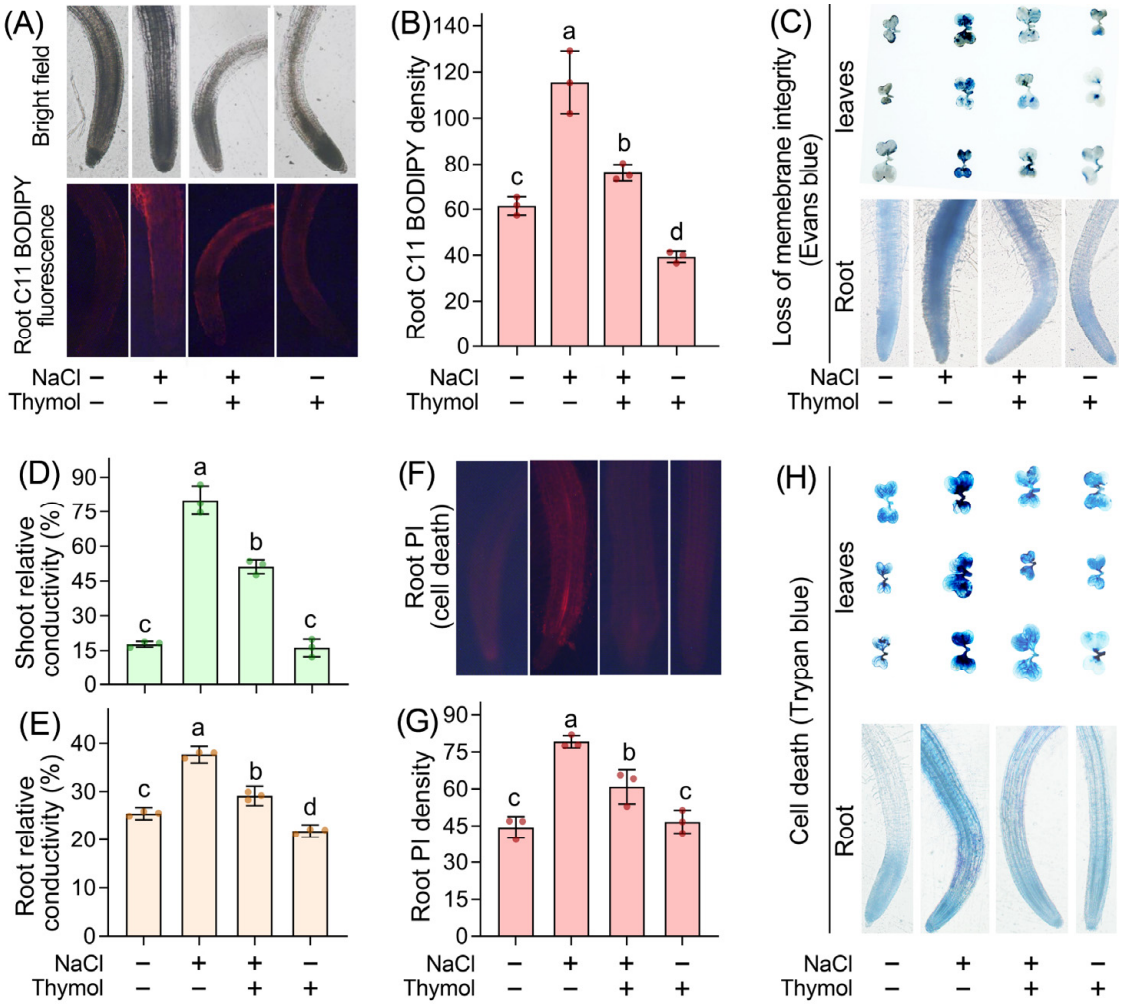

Figure 4. Effects of thymol on oxidative damage and cell death in Chinese cabbage seedlings under saline stress. The seedlings were exposed to NaCl or thymol for 72 h. (**A**) Lipid peroxidation as indicated by C11 BPDIPY fluorescence in roots. (**B**) Relative C11 BPDIPY fluorescent density in roots ($n = 3$). (**C**) Loss of membrane integrity as indicated by Evans blue in leaves (upper panel) and roots (lower panel). (**D**) Relative conductivity in shoots ($n = 3$). (**E**) Relative conductivity in roots ($n = 3$). (**F**) Cell death as indicated by PI fluorescence in roots. (**G**) Relative PI fluorescent density in roots ($n = 3$). (**H**) Cell death as indicated by trypan blue in leaves (upper panel) and roots (lower panel). Different lowercase letters in (**B**,**D**,**E**,**G**) indicate significant differences among different treatments (ANOVA, $p < 0.05$).

3.5. Thymol Activated Antioxidative Enzymes in Chinese Cabbage Seedlings upon Saline Stress

The activity of several antioxidative enzymes was assayed in order to evaluate the effects of thymol on the antioxidative capacity in seedlings under saline stress. The treatment with NaCl for 72 h stimulated the activity of SOD, APX, CAT, and POD in the shoots as compared to the control group. In the shoots, the treatment with thymol + NaCl resulted in a significant increase in the activity of SOD, APX, CAT, and POD by 22.6%, 49.7%, 11.5%, and

10.3%, respectively, as compared to the NaCl treatment (Figure 5A–D). The activity of these enzymes in the roots changed similarly to those of the shoots upon different treatments. In the roots, the treatment with thymol + NaCl resulted in a significant increase in the activity of SOD, APX, CAT, and POD by 14.7%, 17.7%, 59.3%, and 21.7%, respectively, as compared to the NaCl treatment (Figure 5E–H). These results suggest that the antioxidative enzymes were induced upon saline stress. However, the addition of thymol further enhanced their activities in the Chinese cabbage seedlings upon saline stress.

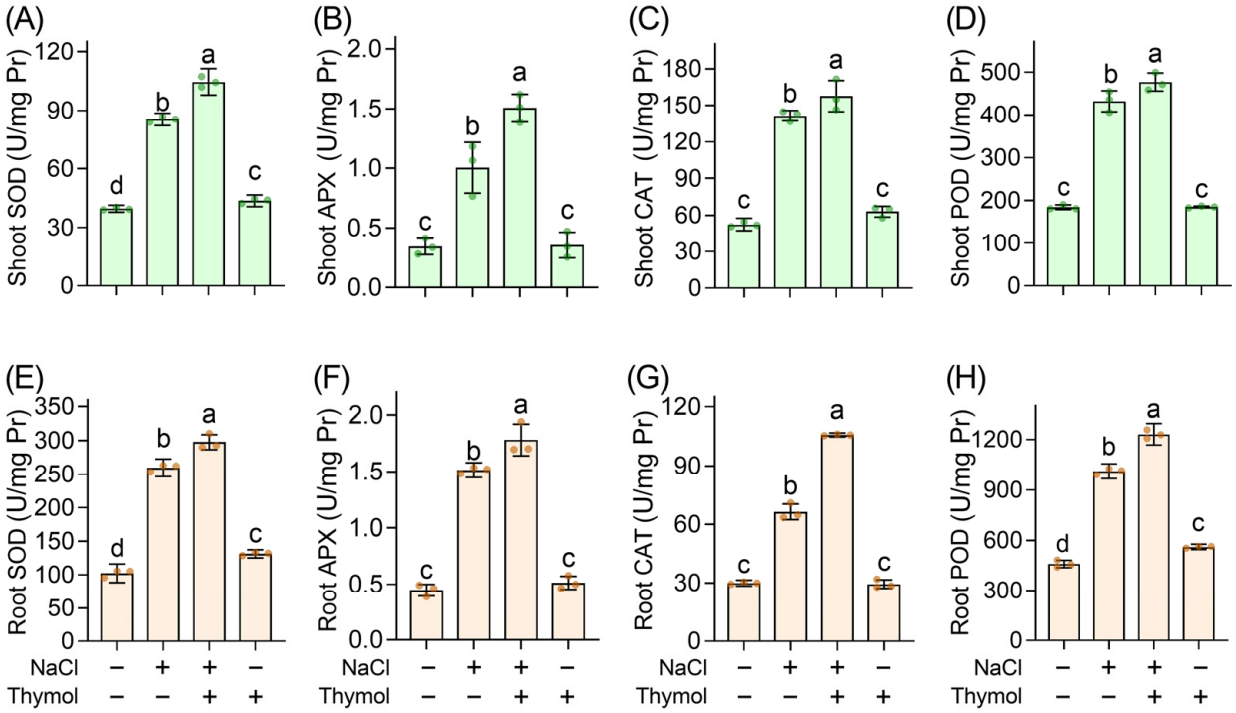

Figure 5. Effects of thymol on the activity of antioxidative enzymes in Chinese cabbage seedlings under saline stress. The seedlings were exposed to NaCl or thymol for 72 h. (**A**–**D**) SOD, APX, CAT, and APX activity in shoots (n = 3). (**E**–**H**) SOD, APX, CAT, and APX activity in roots (n = 3). Different lowercase letters indicate significant differences among different treatments (ANOVA, $p < 0.05$).

3.6. Thymol Adjusted Redox Balance in Chinese Cabbage Seedlings upon Saline Stress

The redox balance in the seedlings was evaluated based on the redox status of AsA and GSH, respectively. In the shoots, the AsA content was decreased by 52.8% upon saline stress (NaCl treatment for 72 h) as compared to the control. The treatment with thymol + NaCl resulted in a significant increase in the AsA content by 72.5% as compared to the NaCl treatment (Figure 6A). The DHA content was enhanced by 36.8% upon saline stress as compared to the control. The treatment with thymol + NaCl resulted in a significant decrease in the DHA content by 8.0% as compared to the NaCl treatment (Figure 6B). AsA/DHA was decreased by 65.5% upon saline stress as compared to the control. The treatment with thymol + NaCl resulted in a significant increase in AsA/DHA by 88.2% as compared to the NaCl treatment (Figure 6C). In the roots, AsA, DHA, and AsA/DHA changed similarly to those of the shoots upon the different treatments (Figure 6D–F). These results suggest that saline stress led to a significant decrease in AsA/DHA by lowering the AsA content and enhancing the DHA content in the Chinese cabbage seedlings.

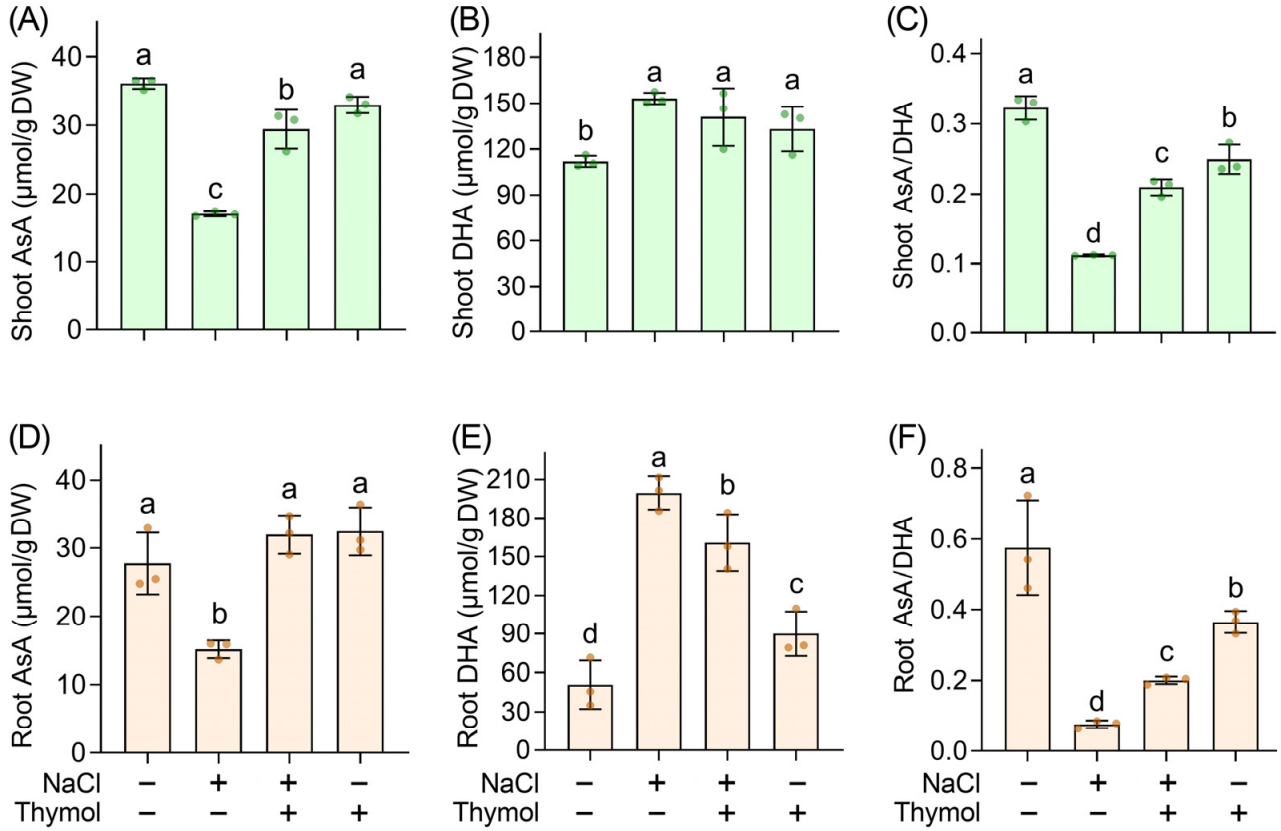

Figure 6. Effects of thymol on the content of AsA and DHA in Chinese cabbage seedlings under saline stress. The seedlings were exposed to NaCl or thymol for 72 h. The upper panel is for shoots (**A**–**C**). The lower panel is for roots (**D**–**F**). Different lowercase letters indicate significant differences among different treatments (n = 3, ANOVA, $p < 0.05$).

In the shoots, the GSH content was decreased by 25.5% upon saline stress (NaCl treatment for 72 h) as compared to the control. The treatment with thymol + NaCl resulted in a significant increase in the GSH content by 114.4% as compared to the NaCl treatment (Figure 7A). The GSSG content was enhanced by 27.6% upon saline stress as compared to the control. The treatment with thymol + NaCl resulted in a significant decrease in the GSSG content by 30.2% as compared to the NaCl treatment (Figure 7B). GSH/GSSG was decreased by 41.2% upon saline stress as compared to the control. The treatment with thymol + NaCl resulted in a significant increase in GSH/GSSG by 207.0% as compared to the NaCl treatment (Figure 7C). In the roots, the treatment with thymol + NaCl increased GSH by 71.4% as compared to the NaCl treatment (Figure 7D). Thymol failed to change the GSSG content in the NaCl-treated roots but led to a significant increase in GSH/GSSG by 80.5% (Figure 7E,F). These results suggest that thymol was able to maintain a reduced cellular status by enhancing AsA/DHA and GSH/GSSG in the Chinese cabbage seedlings upon saline stress.

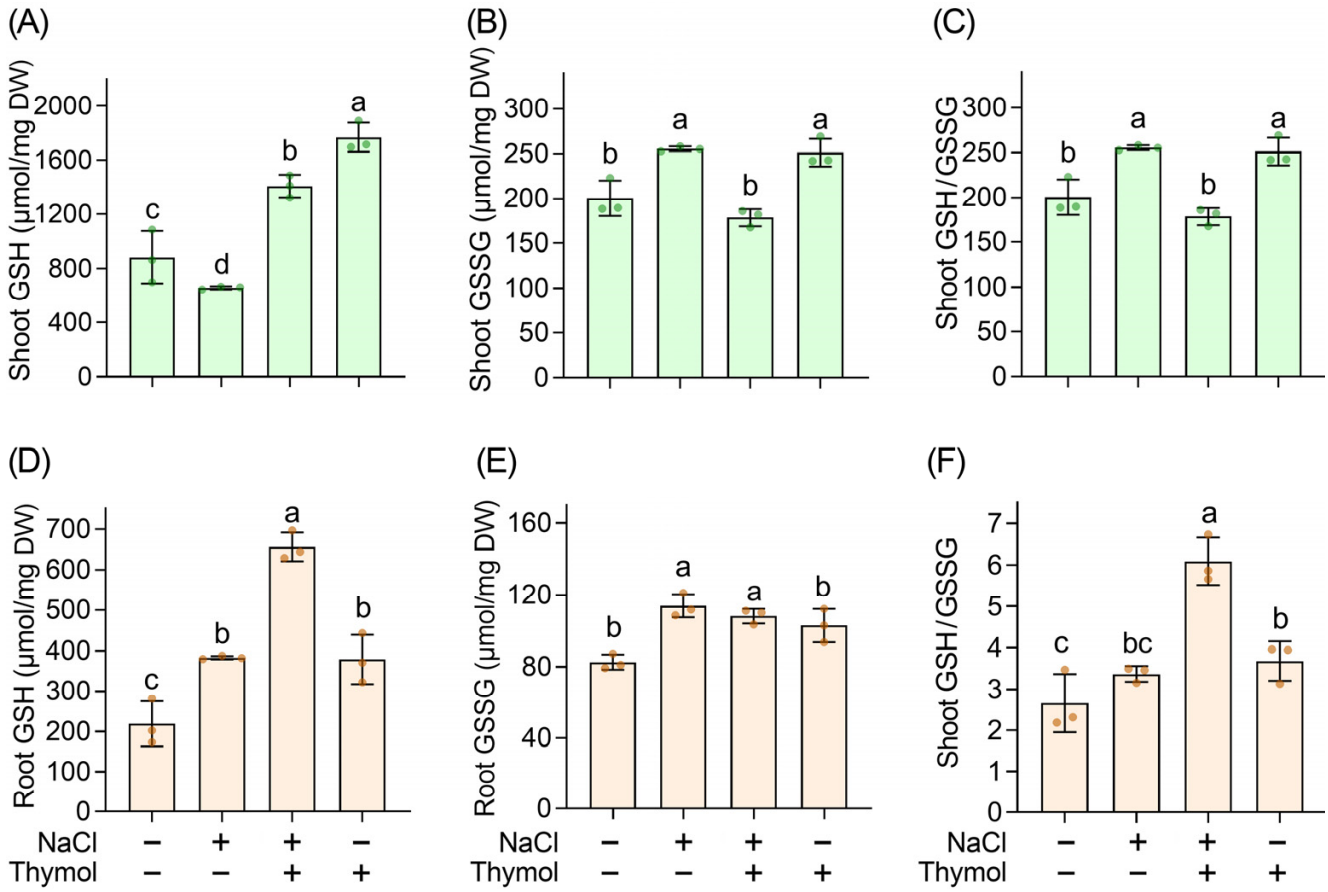

Figure 7. Effects of thymol on the contents of GSH and GSSG in Chinese cabbage seedlings under saline stress. The seedlings were exposed to NaCl or thymol for 72 h. The upper panel is for shoots (**A**–**C**). The lower panel is for roots (**D**–**F**). Different lowercase letters indicate significant differences among different treatments (n = 3, ANOVA, $p < 0.05$).

3.7. Thymol Enhanced Antioxidant Content in Chinese Cabbage Seedlings upon Saline Stress

The shoots had less phenols and flavonoids upon saline stress as compared to the control. The treatment with thymol + NaCl for 72 h resulted in a significant increase in the phenol and flavonoid content by 71.6% and 45.2%, respectively, as compared to the NaCl treatment (Figure 8A,B). Saline stress led to a significant decrease in the soluble protein content in the shoots by 67.5% as compared to the control. However, the treatment with thymol + NaCl resulted in a significant increase in the soluble protein content by 58.0% as compared to the NaCl treatment (Figure 8C). In the roots, we observed similar changes in the phenol, flavonoid, and soluble content upon the NaCl and thymol treatment (Figure 8D–F). These results suggest that thymol enhanced the content of antioxidants in the Chinese cabbage seedlings under saline stress.

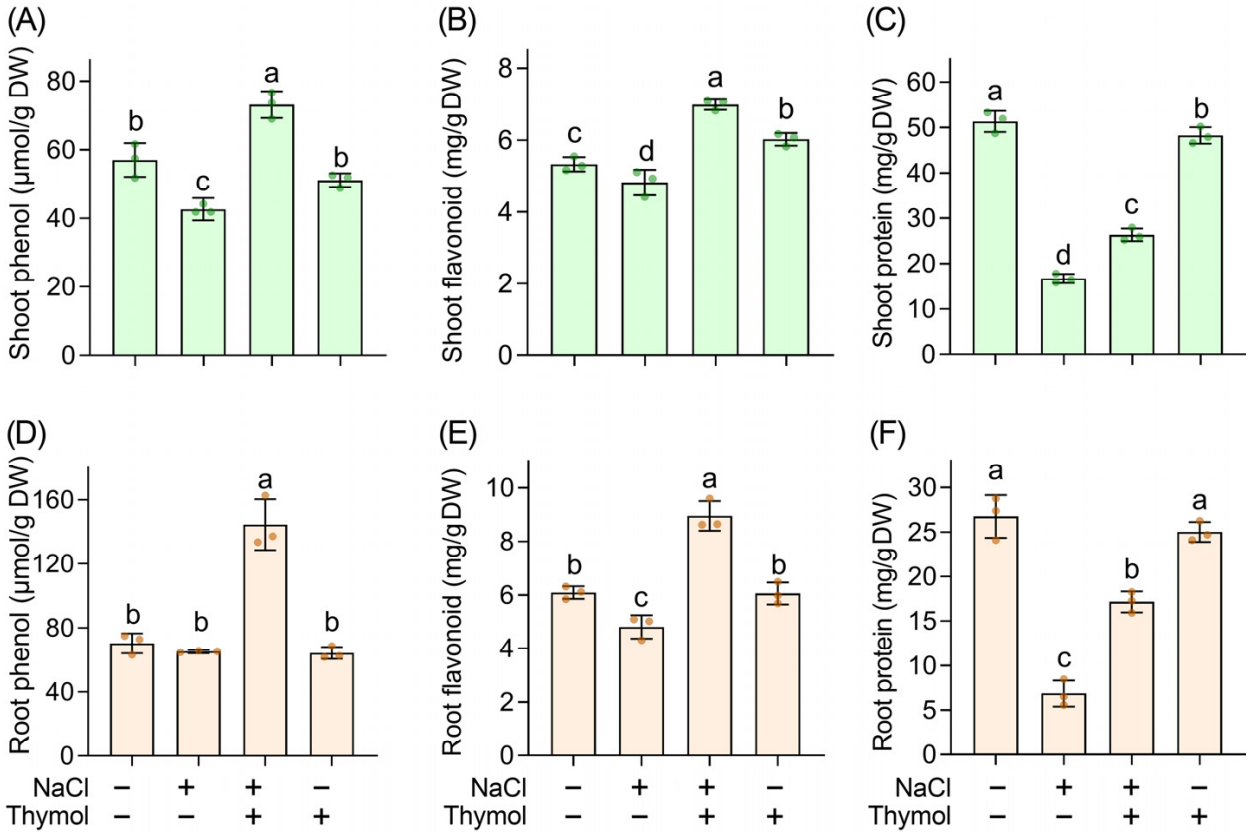

Figure 8. Effects of thymol on phenol, flavonoid, and protein content in Chinese cabbage seedlings under saline stress. The seedlings were exposed to NaCl or thymol for 72 h. (**A**–**C**) Phenol, flavonoid, and protein content in shoots (n = 3). (**D**–**F**) Phenol, flavonoid, and protein content in roots (n = 3). Different lowercase letters indicate significant differences among different treatments (ANOVA, $p < 0.05$).

3.8. Correlation and Cluster Analysis

A Pearson correlation analysis was conducted to evaluate the role of physiological parameters obtained from different treatments (control, NaCl, thymol + NaCl, and thymol) in thymol-induced saline tolerance in the Chinese cabbage seedlings. In the shoots, the fresh weight negatively correlated to DHA and relative conductivity, respectively, but positively correlated to proteins, AsA, and AsA/DHA. The relative conductivity negatively correlated to AsA and AsA/DHA, respectively (left panel in Figure 9). This indicated that the AsA-mediated redox balance played a role in thymol-recovered membrane integrity and shoot growth under saline stress. Four antioxidative enzymes (CAT, SOD, APX, and POD) correlated positively to each other (left panel in Figure 9), suggesting their consistent response to thymol under saline stress.

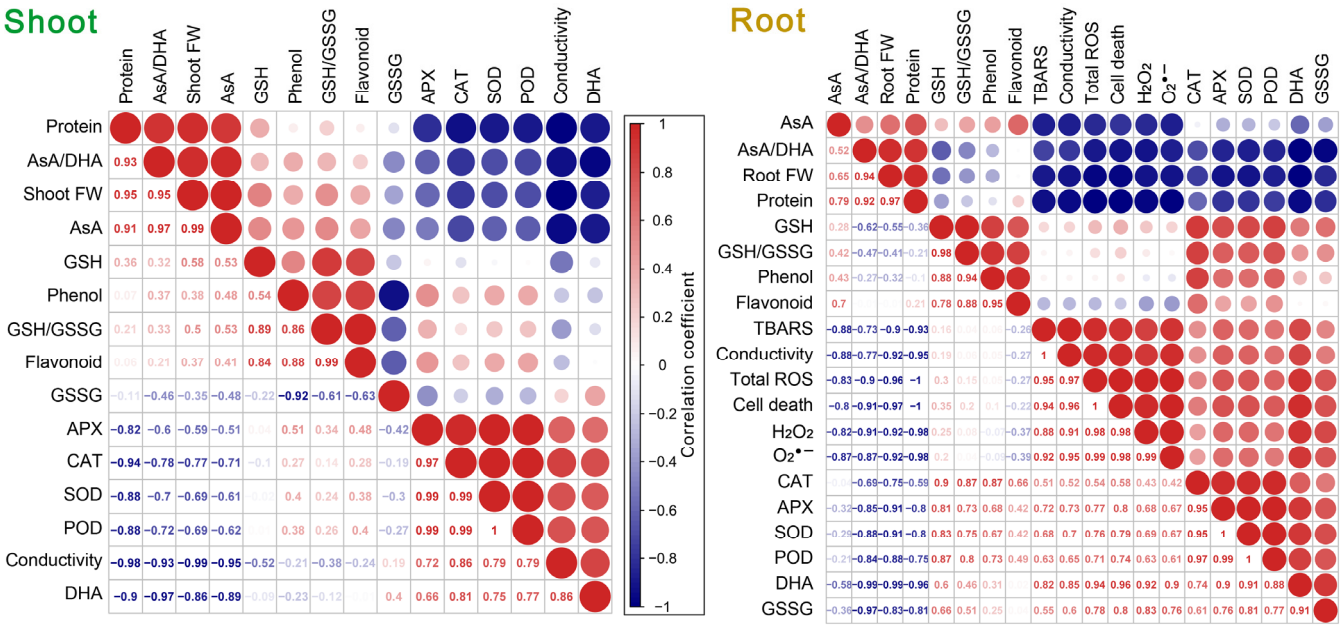

Figure 9. Correlation relationship among different physiological parameters. Physiological parameters obtained from the treatment of control, NaCl (100 mM), NaCl (100 mM) + thymol (20 μM), and thymol (20 μM) were collected to perform Pearson correlation analysis. The left and right panel indicate shoots and roots, respectively. LIP, lipid peroxidation.

In the roots, the fresh weight correlated positively to AsA/DHA and the protein content, respectively. However, the root fresh weight correlated negatively to the injury indexes (e.g., ROS, conductivity, lipid peroxidation, and cell death). The ROS correlated positively to lipid peroxidation, conductivity, and cell death, respectively (right panel in Figure 9). This indicated that ROS caused oxidative stress and cell death, which further caused a retardation of the root growth. All of these injury indexes correlated negatively to AsA and AsA/DHA, respectively (right panel in Figure 9). The phenol and GSH/GSSG correlated positively to antioxidative enzymes (CAT, APX, SOD, and POD) in the roots, suggesting a synergetic role of these antioxidative systems in suppressing ROS accumulation in roots upon thymol + NaCl treatment.

To summarize all the physiological changes, we clustered all the physiological parameters according to their changes upon the treatment of the control, NaCl, and thymol + NaCl. In the shoots, three groups were obtained (Figure 10A). Group I consisted of GSH/GSSG, phenols, GSH, flavonoids, proteins, AsA/DHA, fresh weights, and AsA. Saline stress led to a decrease in these parameters, which was reversed by adding thymol. Group II of consisted GSSG, DHA, and relative conductivity. Saline stress induced an increase in these parameters, which was suppressed by adding thymol. Group III consisted of several antioxidative enzymes, such as CAT, APX, SOD, and POD. Saline stress triggered an increase in these parameters, which was further increased after adding thymol.

In the roots, we also obtained three groups similar to the shoots (Figure 10B). Group I consisted of proteins, fresh weights, AsA/DHA, AsA, phenols, and flavonoids. Saline stress led to a decrease in these parameters, which was increased by further adding thymol. Group II consisted of several injury indexes, such as cell death, ROS, lipid peroxidation, DHA, GSSG, and relative conductivity. Saline stress induced an increase in these parameters, which was suppressed by adding thymol. Group III consisted of four antioxidant enzymes (CAT, APX, SOD, and POD), GSH, and GSH/GSSG. This was similar to Group III for the shoots. These parameters were increased upon salt stress and were increased further after adding thymol.

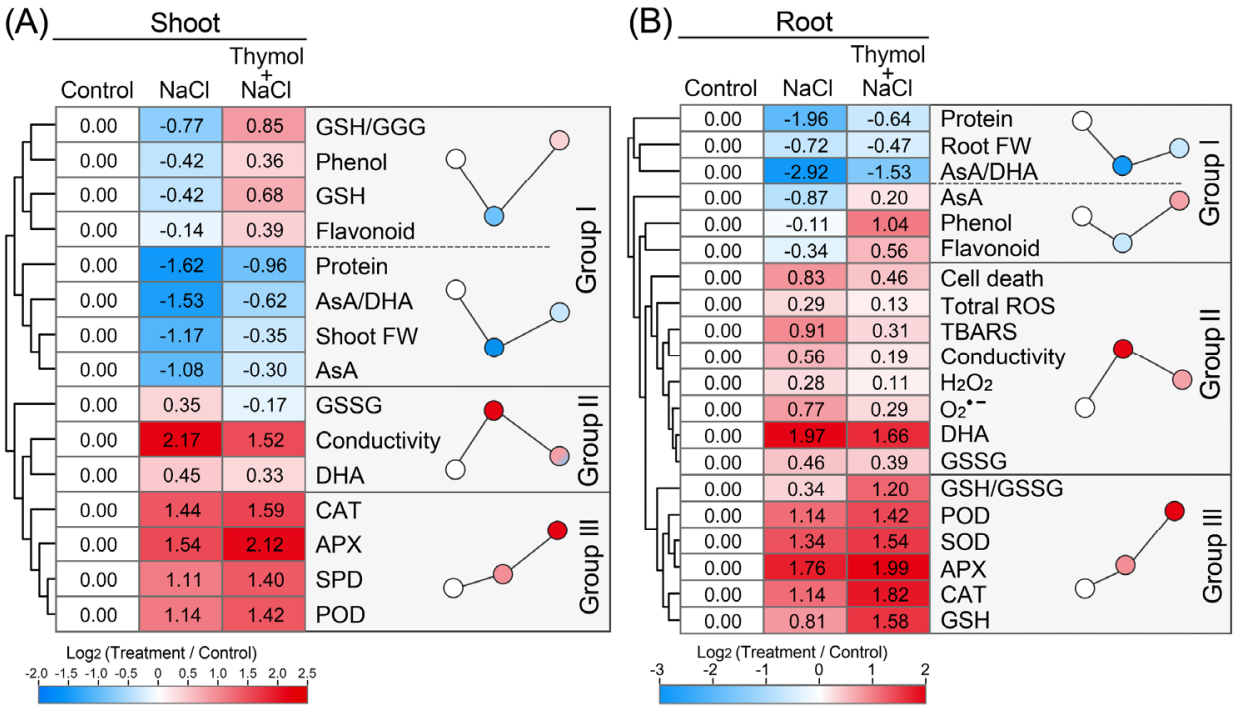

Figure 10. Cluster analysis among different treatments. The physiological parameters were clustered according to their changes upon the treatment of the control, NaCl (100 mM), NaCl (100 mM) + thymol (20 μM). (**A**) Shoots. (**B**) Roots. LIP, lipid peroxidation. The number in each box of the treatments represents the $\log_2$ fold change with respect to the control. For each parameter, red and blue indicate an upregulation and a downregulation, respectively, as compared to the control.

4. Discussion

Developing biostimulants based on natural plant extracts is a promising approach to combat salinity [38]. In this study, we found that thymol had the potential to promote the growth of Chinese cabbage seedlings (just after germination) upon saline stress. This effect was closely associated to the attenuation of oxidative injuries and cell death, which may have resulted from the activation of multiple antioxidative systems in both the roots and the shoots.

Salt-induced ROS can cause lipid peroxidation by attacking membrane lipids, leading to the leakage of intracellular substances [39]. Thymol maintained cell membrane integrity in the salt-treated seedlings, as confirmed by the attenuation of lipid peroxidation and membrane permeability. And this may have directly resulted from the decrease in ROS levels (H_2O_2 and $O_2^{\bullet-}$) in the salt-treated seedlings upon thymol exposure. Thymol attenuated ROS accumulation and the subsequent oxidative damage, helping recover the root growth of the seedlings under saline stress. And three antioxidative systems may be activated by thymol to resist ROS accumulation.

The first system thymol activates consists of several antioxidative enzymes. The increase in endogenous ROS can act as a signal to trigger antioxidative enzymes in plants in response to environmental stimuli [40]. The activities of four enzymes (SOD, POD, CAT, and APX) were enhanced upon saline stress, because the seedlings sensed ROS accumulation. However, it was not enough to combat ROS bursts effectively. Applying thymol further enhanced the activities of these enzymes, which may have helped to lower ROS levels. SOD is considered to be the first defensive line eliminating ROS [41]. Thymol activated SOD, catalyzing $O_2^{\bullet-}$ to the less-toxic H_2O_2, suggesting an effective response of the enzymatic antioxidative system. There are different kinds of SODs in plants, such as FeSOD, Cu-ZnSOD, and MnSOD. These SODs are found to be located in different organelles, such as apoplasts, cytoplasms, mitochondria, and chloroplasts [42]. ROS can be generated in different organelles as well. Thymol may activate different kinds of SODs

to eliminate ROS in different organelles. The H_2O_2 (either produced from SOD or other sources) was further catalyzed into nontoxic H_2O by three other enzymes (CAT, POD, and APX) upon thymol exposure. Thus, thymol controls endogenous ROS levels in seedlings under saline stress by deploying a set of antioxidative enzymes to eliminate over-generated ROS.

The second system induced by thymol involves AsA-DHA and GSH-GSSG. Redox homeostasis is critical for healthy plant cells. ROS can damage cellular redox homeostasis by inducing the oxidized status [43]. AsA provides a hydrophilic redox buffer against oxidative damage in plant cells [44]. Saline stress caused the depletion of AsA by promoting the transformation of AsA to DHA in seedlings, an effect that could be reversed by thymol. This would help maintain the hydrophilic redox buffer inside of cells under saline stress. In addition, AsA acts as a specific electron donor for APX metabolizing H_2O_2 [45]. Thymol-enhanced AsA may provide enough substrates to drive the increase in APX activity, facilitating the elimination of H_2O_2 in saline-stressed seedlings. The positive role of GSH in regulating plant salt tolerance has been genetically identified [46]. Thymol enhances GSH levels to confer saline tolerance, which may work in two ways. The first one is that GSH can help scavenge ROS by coupling AsA to run a GSH-AsA cycle in plants upon saline stress [47]. Secondly, a high GSH/GSSG ration can create a reducing microenvironment inside of cells. This would maintain the functional structure of proteins through *S*-glutathionylation (an important post-translational modification), which protects proteins from being oxidized under stressful conditions. In Arabidopsis, endogenous GSH regulates ethylene signaling to modulate salt tolerance via the *S*-glutathionylation of ACO1 (1-aminocyclopropane-1-carboxylate oxidase 1), a key enzyme for producing ethylene in plants [48].

The third system induced by thymol involves the enhancement of antioxidative secondary metabolites. The ROS-scavenging activity of polyphenols and flavonoids plays a role in plant salt tolerance [49]. The accumulation of thymol and phenolic compounds is important for the enhancement of antioxidant activities in *Thymus vulgaris* and *Thymus daenensis* Celak under saline stress [50]. Here, we found that thymol treatment induced the accumulation of the total phenols and flavonoids in the seedlings under saline stress. Therefore, besides the ROS-scavenging property of thymol itself [51], thymol may also trigger the production of other antioxidative metabolites against saline stress. Phytophenols can support enzymatic antioxidative defenses as well. The antioxidative properties can also be linked to enzymatic or nonenzymatic activities. In plants, polyphenols act as electron donors for guaiacol-type PODs for metabolizing H_2O_2. Polyphenols can also protect plants from oxidative injuries by supporting AsA-dependent antioxidative defenses [52]. Therefore, having link antioxidative enzymes, a redox balance, and antioxidants indicates a multifunctional role of thymol in enhancing the antioxidative capacity in Chinese cabbage seedlings against saline stress.

ROS are multifunctional in the regulation of plant growth and development. Besides triggering oxidative injuries, ROS can manipulate root elongation by regulating cell differentiation. In this mode, H_2O_2 and $O_2^{\bullet-}$ control cell differentiation and proliferation, respectively, in root tips. Normal root elongation can be controlled by the transcription factor UPB1 (UPBEAT1) that maintains a proper balance between H_2O_2 and $O_2^{\bullet-}$ [53]. Abiotic stress (e.g., heavy metal) can inhibit root elongation by disturbing the UPB1-controlled balance between H_2O_2 and $O_2^{\bullet-}$ in root tips [54]. In the present study, saline stress also changed the balance between H_2O_2 and $O_2^{\bullet-}$ in the root tips, which was partially reversed by adding thymol (Figure S1). In mammals, thymol can regulate differentiation and proliferation for immune modulation [55,56], indicating a possible function of thymol in modulating the H_2O_2-$O_2^{\bullet-}$ balance to promote root growth under saline stress.

5. Conclusions

In this study, we identified the biostimulant property of thymol in the regulation of plant salt tolerance from the perspective of antioxidation. In the seedlings of Chinese

cabbage upon saline stress, thymol detoxifies salt-induced oxidative injuries by deploying multiple antioxidative systems, including antioxidative enzymes, antioxidants, and the redox balance (Figure 11). The detailed mechanisms for how thymol integrated these three systems are largely unknown, which leaves several perspectives that need to be studied further. For example, further studying the possible regulation of different kinds of SODs by thymol would help understand thymol-induced saline tolerance in different organelles, especially to differentiate the roots and shoots. The specific role of the redox balance in thymol-induced plant salt tolerance could be explained based on possible protein post-translational modifications. And it would be interesting to explore the mechanisms for thymol facilitating root elongation by modulating the H_2O_2-$O_2^{\bullet-}$ balance in roots under saline stress. Further investigation focusing on the above perspectives would help develop thymol as a potential biostimulant in both applied and fundamental studies.

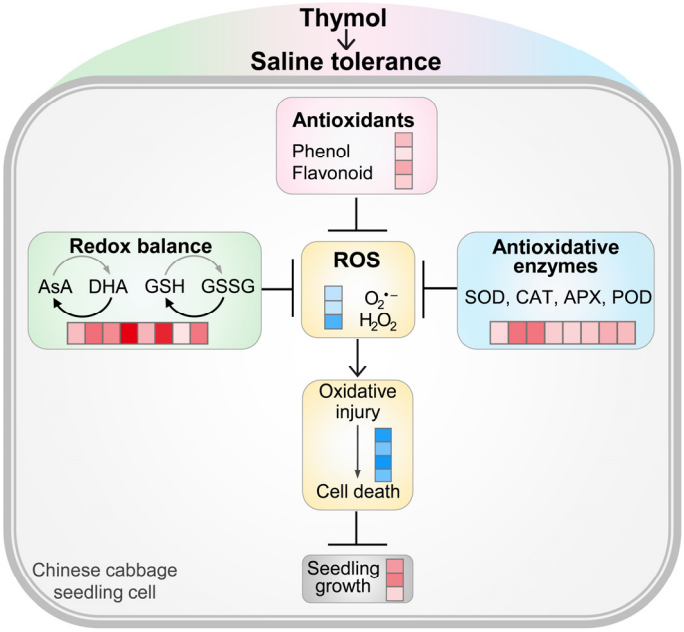

Figure 11. A schematic presentation of thymol-deployed antioxidative systems in Chinese cabbage seedlings under saline stress. The green, pink, and blue panels indicates redox balance, antioxidants, and antioxidative enzymes, respectively, all of which help suppress ROS accumulation. Thymol can activate these three systems. Then, the oxidative injury and cell death can be alleviated, promoting seedling growth under saline stress. The heatmap in each panel indicates the relative change of physiological parameters under treatment of NaCl + thymol as compared to NaCl treatment alone (red, upregulation; blue, downregulation).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14051059/s1, Figure S1: Effects of thymol on H_2O_2/$O_2^{\bullet-}$ in the roots of Chinese cabbage seedlings under saline stress.

Author Contributions: Conceptualization, L.Y., J.C. and X.Y.; methodology, C.S. and L.W.; investigation and data analysis, C.S., J.C., L.W., J.L. and Z.S.; writing—original draft preparation, C.S. and J.C.; writing—review and editing, L.Y. and X.Y. All authors have read and agreed to the published version of the manuscript.

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