The γ-Aminobutyric Acid (GABA) Alleviates Salt Stress Damage during Seeds Germination of White Clover Associated with Na⁺/K⁺ Transportation, Dehydrins Accumulation, and Stress-Related Genes Expression in White Clover

Bizhen Cheng †, Zhou Li †, Linlin Liang, Yiqin Cao, Weihang Zeng, Xinquan Zhang, Xiao Ma, Linkai Huang ‡, Gang Nie, Wei Liu and Yan Peng *

Department of Grassland Science, College of Animal Science and Technology, Sichuan Agricultural University, Chengdu 611130, China; Chengbizhenggrass@163.com (B.C.); lizhou1986814@163.com (Z.L.); LiangllCLJ@163.com (L.L.); c545755756@163.com (Y.C.); zengwh0123@163.com (W.Z.); zhangxq@sicau.edu.cn (X.Z.); maroar@126.com (X.M.); huanglinkai@sicau.edu.cn (L.H.); nieganggrass@hotmail.com (G.N.); lwgrass@126.com (W.L.)

* Correspondence: pengyanlee@163.com
† These authors contributed equally to this work.

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Abstract: The objective of this study was to determine the effect of soaking with γ-aminobutyric acid (GABA) on white clover (Trifolium repens cv. Haifa) seed germination under salt stress induced by 100 mM NaCl. Seeds soaking with GABA (1 µM) significantly alleviated salt-induced decreases in endogenous GABA content, germination percentage, germination vigor, germination index, shoot and root length, fresh and dry weight, and root activity of seedling during seven days of germination. Exogenous application of GABA accelerated starch catabolism via the activation of amylase and also significantly reduced water-soluble carbohydrate, free amino acid, and free proline content in seedlings under salt stress. In addition, improved antioxidant enzyme activities (SOD, GPOX, CAT, APX, DHAR, GR and MDHR) and gene transcript levels (Cu/ZnSOD, FeSOD, MnSOD, CAT, GPOX, APX, MDHR, GPX and GST) was induced by seeds soaking with GABA, followed by decreases in O₂·−, H₂O₂, and MDA accumulation during germination under salt stress. Seeds soaking with GABA could also significantly improve Na⁺/K⁺ content and transcript levels of genes encoding Na⁺/K⁺ transportation (HKT1, HKT8, HAL2, H⁺-ATPase and SOS1) in seedlings of white clover. Moreover, exogenous GABA significantly induced the accumulation of dehydrins and expression of genes encoding dehydrins (SK2, Y2K, Y2SK, and dehydrin b) in seedlings under salt stress. These results indicate that GABA mitigates the salt damage during seeds germination through enhancing starch catabolism and the utilization of sugar and amino acids for the maintenance of growth, improving the antioxidant defense for the alleviation of oxidative damage, increasing Na⁺/K⁺ transportation for the osmotic adjustment, and promoting dehydrins accumulation for antioxidant and osmotic adjustment under salt stress.

Keywords: growth; osmotic adjustment; metabolism; dehydrins; antioxidant; transcript

1. Introduction

It has been reported that over 900 million hectares of land is negatively affected by salinity, accounting for 7% of the world’s total land [1]. Salinity has become a major problem limiting the growth and development of various plants. Effects of salt damage on plants are involved in multiple
morphological, physiological, and biochemical processes [2]. For example, plant roots could be negatively affected by high concentration of Na\textsuperscript{+} and Cl\textsuperscript{−}. The overaccumulation of Na\textsuperscript{+} and Cl\textsuperscript{−} in plants causes ion toxicity and also decreases the absorption of other ions leading to growth inhibition and metabolic disturbance under salt stress. In addition, salt stress seriously reduces the ability of plants taking up water resulting in physiological drought [3–6]. Those negative effects directly or indirectly lead to the overaccumulation of reactive oxygen species (ROS) which cause oxidative damage in plants under salt stress [7]. It is well-known that salt stress negatively affects all vegetative stages of plants, especially for seed germination [8]. Seed germination is the most important and sensitive process during plant growth and development. Previous studies have indicated that salt stress significantly reduced germination percentage, germination vigor, shoot and root length of different plant species [9–11].

The γ-aminobutyric acid (GABA) is a natural nonprotein amino acid that exists in both animals and plants. GABA has been widely studied as an important inhibitory neurotransmitter in animals [12]. In plants, GABA is associated with the maintenance of the carbon-nitrogen balance, the metabolism of amino acids and carbohydrates, and the regulation of growth and cell pH [13]. GABA is also involved in plants response to abiotic stresses, diseases, and insects [14–16]. Some studies have shown that the accumulation of GABA rapidly increased when plants were exposed to many adverse conditions, such as hypoxia, drought, cold, high temperature, low light, and high salt, indicating GABA could play a major role during abiotic stresses [14]. It has been reported that the GABA in plant cells could act as an effective osmolyte without toxic effects during the salt-induced dehydration and had the function of ROS scavenging under stressful environmental condition [17]. In addition, it has been found that the suitable concentration of exogenous GABA could promote plant growth, antioxidant metabolism, and transcript levels of genes encoding antioxidant enzymes, thereby alleviating stress-caused oxidative damage in plants [14,15]. Exogenous application of GABA could also regulate the osmotic balance in plant cells contributing to the enhancement of stress tolerance [18–20]. More importantly, exogenous GABA effectively inhibited the production of H\textsubscript{2}O\textsubscript{2} and reduced oxidative damage through regulating the expression of key genes of H\textsubscript{2}O\textsubscript{2} production and genes encoding peroxidases in Caragana intermedia roots under salt stress [14]. Seed soaking with GABA also significantly increased seed germination percentage and reduced the salt-caused injury during seeds germination of wheat and maize [21], but the possible mechanism still needs to be further investigated.

As an important legume forage with high content of crude protein, white clover (Trifolium repens L.) is widely cultivated all over the world, but it is susceptible to salt stress [22,23]. As mentioned above, previous studies have proved that exogenous GABA could significantly improve the tolerance to abiotic stresses in various plant species, but little information is available about GABA-regulated salt tolerance during seeds germination. The purposes of this study were (1) to examine effects of seeds soaking with the GABA on germination characteristics and (2) to reveal GABA-regulated salt tolerance associated with antioxidant defense, osmotic adjustment, Na\textsuperscript{+}/K\textsuperscript{+} transportation, dehydrins accumulation, and relevant genes expression during seeds germination of white clover under salt stress. The study will largely contribute to further understanding salt-tolerant mechanism induced by GABA in plants.

2. Results

2.1. Effects of the GABA on Seed Germination Characteristics

The germination vigor (GV), germination percentage (GP), germination index (GI), mean germination time (MGT), and seed vigour index (VI) significantly decreased in response to salt stress. Exogenous GABA did not have significant effects on GV, GP, GI, MGT, and VI under the normal water condition. The low concentration (0.5 and 1 µM) of GABA significantly improved GV, GP, GI, and VI, whereas high concentration (5 mM) of GABA inhibited seeds germination of white clover under salt stress (Table 1). Under salt stress, seeds primed with 0.5 and 1 µM GABA had 6.5%
and 11.77% higher GP than seeds primed with water, respectively (Table 1). Under normal water condition, seeds soaking with the different concentration of GABA did not have significant effects on seedlings fresh weight (FW), dry weight (DW), root length (RL), shoot length (SL), and shoot-root ratio (Table 2). Under salt stress, seedling FW, DW, RL, SL and shoot-root ratio significantly decreased. Seeds priming with exogenous GABA (1 µM) had significantly higher seedlings DW, longer SL, and shoot-root ratio than seeds priming with water in response to salt stress (Table 2). Similarly, seeds soaking with lower concentration of GABA (0.5, 1, 2.5 µM) maintained significantly higher seedlings FW than seeds priming with water under salt stress (Table 2).

2.2. Effect of the GABA on Root Activity and Endogenous GABA Content

Figure 1A showed the phenotypic difference between seeds soaking with GABA and water after seven days of germination under normal and salt stress conditions. The root activity and endogenous GABA content of seedlings between seeds soaking with GABA and water did not show significant difference under normal water condition, but seeds soaking with GABA exhibited significantly higher root activity than seeds soaking with water under salt stress. The root activity and endogenous GABA content of seedlings with GABA treatment increased by 21.6 and 62.2% compared to the seedlings without GABA treatment under salt stress, respectively (Figure 1B,C).

![Figure 1](image)

**Figure 1.** Effects of seed soaking with GABA or water on (A) phenotypic changes, (B) root activity, and (C) endogenous GABA content during seeds germination (7 days) under salt stress. Vertical bars indicate ± SE of mean (n = 6). Different letters above indicate significant difference. LSD (p ≤ 0.05).

2.3. Effects of the GABA on Starch Metabolism and Osmotic Adjustment

Seeds soaking with GABA had no significant impact on starch content and amylase activity under the normal water condition (Figure 2A–D). In response to salt stress, the α-amylase activity was significantly inhibited without GABA pretreatment. However, the seeds soaking with GABA significantly improved total, α-, and β-amylase activity and significantly decreased starch content (Figure 2A–D). There are no significant differences on osmotic potential (OP), soluble sugar, free amino acid, and free proline content between seeds priming with and without GABA under normal water condition (Figure 3A–D). The salt stress significantly increased soluble sugar and free proline content, but decreased OP in seedlings. Seedlings with GABA treatment had distinctly lower soluble sugar, amino acids (AA), and free proline content than seedlings without GABA treatment under salt stress (Figure 3A–D).
Table 1. Effects of seed priming with water or γ-aminobutyric acid (GABA) on seed germination characteristics in white clover under seven days of different salt stress conditions. Values are mean ± standard error (SE) (n = 6). Different letters in a vertical column indicate a significant difference between each treatment under different NaCl concentration. The asterisk (*) indicates a significant difference exists between seed priming with water or GABA. LSD (p ≤ 0.05).

| GABA (µM) | Germination Percentage (%) | Germination Vigor (%) | Germination Index | Mean Germination Time (d) | Seed Vigour Index |
|-----------|----------------------------|-----------------------|-------------------|---------------------------|------------------|
|           | Water | NaCl | Water | NaCl | Water | NaCl | Water | NaCl | Water | NaCl |
| 0.00      | 95.33 ± 1.15 * | 51.50 ± 1.00 c,d           | 90.80 ± 1.10 a     | 33.33 ± 2.31 b,c           | 35.66 ± 1.28 a     | 9.29 ± 0.75 c           | 1.61 ± 0.05 a     | 3.28 ± 0.29 **      | 1.58 ± 0.13 a       | 0.34 ± 0.02 d     |
| 0.25      | 96.00 ± 1.00 a   | 54.67 ± 1.15 a,b           | 93.20 ± 1.10 a     | 40.67 ± 1.15 a             | 35.66 ± 1.47 a     | 10.46 ± 0.16 b           | 1.63 ± 0.02 a     | 3.10 ± 0.21 **      | 1.59 ± 0.04 a       | 0.38 ± 0.01 c     |
| 0.50      | 95.60 ± 1.67 a   | 58.00 ± 5.20 ab            | 93.33 ± 1.15 a     | 39.33 ± 3.06 ab            | 35.86 ± 1.97 a     | 11.05 ± 0.88 ab          | 1.85 ± 0.52 a     | 3.07 ± 0.14 **      | 1.59 ± 0.08 a       | 0.41 ± 0.03 b     |
| 1.00      | 96.00 ± 1.41 a   | 63.33 ± 4.16 ab            | 93.50 ± 1.00 a     | 42.00 ± 2.00 ab            | 34.67 ± 3.00 a     | 11.73 ± 0.11 ab          | 1.75 ± 0.15 a     | 3.06 ± 0.12 **      | 1.53 ± 0.18 a       | 0.45 ± 0.01 ab    |
| 2.50      | 96.00 ± 2.83 a   | 50.50 ± 4.43 d             | 92.50 ± 1.91 a     | 32.67 ± 1.15 b             | 33.92 ± 1.57 a     | 9.15 ± 0.29 ab           | 1.75 ± 0.09 a     | 3.07 ± 0.09 **      | 1.48 ± 0.09 a       | 0.35 ± 0.01 d     |
| 5.00      | 95.60 ± 0.90 a   | 46.00 ± 4.90 d             | 92.50 ± 1.91 a     | 32.00 ± 3.46 b             | 34.19 ± 2.15 a     | 8.77 ± 0.26 ab           | 1.68 ± 0.06 a     | 3.12 ± 0.19 a       | 1.51 ± 0.11 a       | 0.32 ± 0.01 d     |

Table 2. Effects of seed priming with water or GABA on seed germination characteristics in white clover under seven days of different salt stress conditions. Values are mean ± SE (n = 6). Different letters in a vertical column indicate a significant difference between each treatment under different NaCl concentration. The asterisk (*) indicates a significant difference exists between seed priming with water or GABA. LSD (p ≤ 0.05).

| GABA (µM) | Seedling Fresh Weight (mg/10 Seedling⁻¹) | Seedling Dry Weight (mg/10 Seedling⁻¹) | Root Length (cm) | Shoot Length (cm) | Shoot-Root Ratio |
|-----------|------------------------------------------|----------------------------------------|------------------|-------------------|------------------|
|           | Water | NaCl | Water | NaCl | Water | NaCl | Water | NaCl | Water | NaCl |
| 0.00      | 44.26 ± 2.44 a | 36.25 ± 0.96 c,b     | 3.36 ± 0.15 a     | 3.08 ± 0.2 b,c     | 1.03 ± 0.09 a     | 0.21 ± 0.02 c     | 0.35 ± 0.05 a     | 0.25 ± 0.01 b,a    | 2.85 ± 0.23 a     | 0.85 ± 0.11 b,c   |
| 0.25      | 45.38 ± 0.88 a | 36.30 ± 0.70 b,c     | 3.33 ± 0.21 a     | 3.18 ± 0.2 a       | 1.07 ± 0.10 a     | 0.24 ± 0.01 b     | 0.37 ± 0.02 a     | 0.27 ± 0.02 b,a    | 2.80 ± 0.30 a     | 0.87 ± 0.06 b,c   |
| 0.50      | 45.58 ± 3.24 a | 37.33 ± 0.60 b,c     | 3.30 ± 0.20 a     | 3.13 ± 0.13 ab     | 1.10 ± 0.06 a     | 0.23 ± 0.01 b,c    | 0.36 ± 0.02 a     | 0.27 ± 0.01 b,a    | 2.98 ± 0.08 a     | 0.87 ± 0.09 b,c   |
| 1.00      | 44.82 ± 1.78 a | 37.97 ± 0.87 a       | 3.35 ± 0.10 a     | 3.45 ± 0.13 c      | 1.06 ± 0.06 a     | 0.29 ± 0.04 a     | 0.37 ± 0.02 a     | 0.30 ± 0.02 a       | 2.85 ± 0.17 a     | 0.98 ± 0.10 a     |
| 2.50      | 42.68 ± 0.81 a | 37.77 ± 0.59 a       | 3.33 ± 0.12 a     | 3.15 ± 0.26 ab     | 1.00 ± 0.01 a     | 0.26 ± 0.04 ab     | 0.37 ± 0.02 a     | 0.26 ± 0.02 a       | 2.77 ± 0.12 a     | 0.87 ± 0.11 a     |
| 5.00      | 42.80 ± 1.77 a | 36.07 ± 0.58 a       | 3.26 ± 0.06 a     | 2.83 ± 0.21 c      | 1.01 ± 0.03 a     | 0.21 ± 0.02 c     | 0.37 ± 0.01 a     | 0.26 ± 0.01 b,a    | 2.88 ± 0.30 a     | 0.76 ± 0.05 b,c   |
Figure 2. Effects of seed soaking with GABA or water on (A) starch content, (B) amylase activity, (C) α-amylase activity, and (D) β-amylase activity during seeds germination (7 days) under salt stress. Vertical bars indicate ± SE of mean (n = 6). Different letters above indicate significant difference. LSD (p ≤ 0.05).

Figure 3. Effects of seed soaking with GABA or water on (A) osmotic potential, (B) soluble sugar content, (C) free amino acid content, and (D) free proline content during seeds germination (7 days) under salt stress. Vertical bars indicate ± SE of mean (n = 6). Different letters above indicate significant difference. LSD (p ≤ 0.05).

The GABA treatment did not have an influence on Na⁺ content, but it significantly reduced the K⁺ content under normal water condition (Figure 4A,B). The salt stress significantly increased the
Na⁺ content and reduced the K⁺ content (Figure 4A,B). When seeds pretreated with GABA, the Na⁺ and K⁺ content in seedlings significantly increased under salt stress (Figure 4A,B). Under normal water condition, the treatment of seeds soaking with GABA had no effects on transcript levels of genes involved in Na⁺/K⁺ transporter (VP1, HKT1, HKT8, SKOR, HAL2, H⁺-ATPase, SOS1, NHX6) (Figure 4C). The salt stress significantly improved the VP1 transcript levels and significantly inhibited transcript levels of HKT1, HKT8, HAL2, H⁺-ATPase, SOS1, NHX6 in seedlings without GABA treatment, but did not inhibit these genes expression in seedlings with GABA treatment (Figure 4C). Under salt stress, transcript levels of HKT1, HKT8, HAL2, H⁺-ATPase and SOS1 in seeds soaking with GABA is 8.28, 9.57, 7.04, 2.90, and 2.40 times higher than that in seeds soaking with water (Figure 4C).

Figure 4. Effects of seed soaking with GABA or water on (A) total sodium content, (B) total potassium content, and (C) Sodium/potassium transporter gene (VP1, HKT1, HKT8, SKOR, HAL2, H⁺-ATPase, SOS1, NHX6) relative expression ratio during seeds germination (7 days) under salt stress. Vertical bars indicate ± SE of mean (n = 4). Different letters above columns indicate significant difference. LSD (p ≤ 0.05).

2.4. Effects of the GABA on Antioxidant Defense and Oxidative Damage

The superoxide anion (O₂⁻), perhydrol (H₂O₂), malondialdehyde (MDA) content, and electrolyte leakage (EL) did not show significant differences between GABA-treated or water-treated seedlings under normal water condition (Figure 5A–D). The salt stress obviously raised O₂⁻, H₂O₂, MDA content, and EL, but seeds soaking with GABA significantly declined O₂⁻, H₂O₂, MDA content, and EL (Figure 5A–D). All detected antioxidant enzyme activities did not show significant differences between seedlings with and without the GABA priming under normal water condition (Figure 6A). The salt stress evidently improved superoxide dismutase (SOD), guaiacol peroxidase (GPOX), catalase (CAT), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and monodehydroascorbate reductase (MDHR) activities, but remarkably inhibited ascorbate peroxidase (APX) activity (Figure 6A). The treatment of GABA significantly improved SOD, GPOX, CAT, APX and MDHR activities under salt stress (Figure 6A). Under normal water condition, exogenous GABA only up-regulated glutathione S-transferase (GST) and glutathione peroxidase (GPX) gene expression (Figure 6B).
salt stress significantly decreased transcript levels of ascorbate peroxidase gene (APX), GST, and GPX (Figure 5B). Seeds soaking with GABA evidently improved transcript levels of superoxide dismutase genes (Cu/ZnSOD, MnSOD, FeSOD), guaiacol peroxidase gene (GPX), catalase gene (CAT), APX, monodehydro ascorbate reductase gene (MDHR), GST and GPX under salt stress. The transcript levels of Cu/ZnSOD, MnSOD, FeSOD, GPX, CAT, MDHR, GST and GPX in seedlings with GABA treatment is 2.47, 5.81, 5.58, 10.83, 3.54, 2.94, 7.54, and 47.56 times higher than that in seedlings without GABA treatment under salt stress, respectively (Figure 5B).

2.5. Effects of the GABA on Accumulation of Dehydrins and Genes Relative Expression

Seeds soaking with GABA had no influence on the abundance of dehydrins (65 KDa) during germination under non-stress condition. The salt stress significantly increased the abundance of dehydrins, and the GABA treatment further enhanced the accumulation of dehydrins during germination under salt stress (Figure 7A,B). Under normal water condition, GABA had no significant influence on transcript levels of dehydrins genes (SK2, Y2K, Y2SK and dehydrin b), but evidently improved SK2 expression (Figure 7C). The salt stress significantly improved Y2K and Y2SK transcript levels, but inhibited dehydrin b expression (Figure 7C). During germination, seeds soaking with GABA exhibited significantly higher SK2, Y2K, Y2SK and dehydrin b transcript levels than seeds soaking with water under salt stress. Transcript levels of SK2, Y2K, Y2SK, or dehydrin b in seedlings with GABA treatment is 2.71, 4.58, 3.42, or 142.19 times as high as transcript levels of these genes in seedlings without GABA treatment under salt stress, respectively (Figure 7C). Principal component analysis (PCA) showed that two principal components explained and predicted 99.29% of the total variance (Figure 8). A distinct separation was obtained among “Water and GABA”, “NaCl”, and “GABA+NaCl”, which indicates that GABA had significant effects on seed germination under salt stress (Figure 8).

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Effects of seed soaking with GABA or water on (A) superoxide anion (O$_2^-$) content, (B) perhydrol (H$_2$O$_2$) content, (C) malondialdehyde (MDA) content, and (D) electrolyte leakage (EL) during seeds germination (7 days) under salt stress. Vertical bars indicate ±SE of mean (n = 6). Different letters above indicate significant difference. LSD (p ≤ 0.05).
Figure 6. Effects of seed soaking with GABA or water on (A) antioxidant enzyme activity and (B) gene relative expression ratio during seeds germination (7 days) under salt stress. SOD, superoxide dismutase activity; CAT, catalase activity; GPOX, guaiacol peroxidase activity; APX, ascorbate peroxidase activity; DHAR, dehydroascorbate reductase activity; GR, glutathione reductase activity; MDHR, monodehydroascorbate reductase activity. Cu/ZnSOD, FeSOD and MnSOD, superoxide dismutase genes; CAT, catalase gene; GPOX, guaiacol peroxidase gene; APX, ascorbate peroxidase gene; DHAR, dehydroreductase gene; CytGR, glutathione reductase gene; MDHR, monodehydroascorbate reductase gene; GPX, glutathione peroxidase gene; GST, glutathione S-transferase gene. Vertical bars indicate ± SE of mean (n = 4). Different letters above columns indicate significant difference. LSD (p ≤ 0.05).

Figure 7. Effects of seed soaking with GABA or water on (A) and (B) dehydrins abundance and (C) dehydrins genes (SK2, Y2K, Y2SK and dehydrin b) relative expression ratio during seeds germination (7 days) under salt stress. Vertical bars indicate ± SE of mean (n = 4). Different letters above columns indicate significant difference. LSD (p ≤ 0.05).
provides available carbohydrates for seeds germination and growth. Previous studies have found that the percentage of white clover seeds drop to half of normal germination percentage, indicating that seeds were also critical for the maintenance of cell turgor and energy sources when seeds are subjected to osmotic and ionic stress [29]. Our results show that the salt stress significantly reduced the germination of white clover, but this effect is dependent on appropriate concentration of GABA.

During seeds germination, the starch catabolism is a primary importance because the process provides available carbohydrates for seeds germination and growth. Previous studies have found that environmental stresses such as drought and salt decreased seeds germination associated with the inhibition of starch catabolism [28,29]. Metabolites of starch catabolism, such as soluble sugar, are also critical for the maintenance of cell turgor and energy sources when seeds are subjected to osmotic and ionic stress [29]. Our results show that the salt stress significantly reduced the starch catabolism and utilization of carbohydrate in seeds soaking with H$_2$O, but accelerated starch catabolism through activating α- and β-amylase activities in GABA-pretreated seeds. Interestingly, although seeds soaking with H$_2$O had significantly higher organic osmolytes (sugar and proline) than seeds soaking with GABA during germination, salt stress decreased osmotic potential of these two treatments to the same level. As compared to untreated seeds, GABA pretreatment improved Na$^+$/K$^+$ transportation and the accumulation of Na$^+$ during germination under salt stress. It is well-known that both organic and inorganic osmolytes are important osmotic regulators for plants adaption to harsh environmental stress [30–32]. Our earlier study also found that low concentration of exogenous Na$^+$ (30 mM) pretreatment could significantly enhance osmotic adjustment of white clover associated with salt stress (Figure 8).

### 3. Discussion

The high concentration of salt limits the growth and yield of many crops including white clover [23–26]. The study of Barbagallo et al. showed that salt stress significantly reduced the GP, GV, and VI of canola seeds [9]. During seeds germination, the salt stress also had obviously negative effects on SL, RL, FW, and DW leading to declines in growth and yield of canola [9]. It has been found that the exogenous GABA could significantly improve the SL, RL, and FW of maize (*Zea mays* L.) seedling under salt stress [27]. In our study, the 100 mM NaCl-induced salt stress made the germination percentage of white clover seeds drop to half of normal germination percentage, indicating that seeds germination of white clover was highly sensitive to salt. Seeds soaking with lower concentration of GABA (0.25, 0.5, and 1 µM) effectively alleviated salt-caused inhibition of germination characteristics and 1 µM of GABA exhibited best effects on the improvement of GP, GV, GI, and VI. In addition, seeds soaking with 1 µM of GABA could also significantly increase SL, RL, FW, and DW of seedlings under salt stress. However, higher concentration of GABA (5 µM) inhibited seeds germination of white clover under salt stress. These results suggest that GABA could significantly improve salt tolerance of white clover seeds, but this effect is dependent on appropriate concentration of GABA.

Figure 8. Principal component analysis (PCA) based on analyzed parameters. Each dot indicates each replicate of each treatment. Four treatments were showed in PCA including “Water”, “GABA”, “NaCl”, and “GABA+NaCl”.

During seeds germination, the starch catabolism is a primary importance because the process provides available carbohydrates for seeds germination and growth. Previous studies have found that environmental stresses such as drought and salt decreased seeds germination associated with the inhibition of starch catabolism [28,29]. Metabolites of starch catabolism, such as soluble sugar, are also critical for the maintenance of cell turgor and energy sources when seeds are subjected to osmotic and ionic stress [29]. Our results show that the salt stress significantly reduced the starch catabolism and utilization of carbohydrate in seeds soaking with H$_2$O, but accelerated starch catabolism through activating α- and β-amylase activities in GABA-pretreated seeds. Interestingly, although seeds soaking with H$_2$O had significantly higher organic osmolytes (sugar and proline) than seeds soaking with GABA during germination, salt stress decreased osmotic potential of these two treatments to the same level. As compared to untreated seeds, GABA pretreatment improved Na$^+$/K$^+$ transportation and the accumulation of Na$^+$ during germination under salt stress. It is well-known that both organic and inorganic osmolytes are important osmotic regulators for plants adaption to harsh environmental stress [30–32]. Our earlier study also found that low concentration of exogenous Na$^+$ (30 mM) pretreatment could significantly enhance osmotic adjustment of white clover associated with salt stress (Figure 8).
with increases in the Na⁺ absorption and transportation instead of accumulating more carbohydrates and proline [22]. Previous studies have found that Na⁺ sequestration in the vacuole could act as a cheaper osmoregulatory solutes than organic osmolytes because the synthesis and accumulation of organic osmolytes costs more energy in plant cells [33,34]. Our current findings indicated that the increase in starch catabolism induced by GABA mainly provide available carbohydrates for seeds germination and growth of white clover instead of going to osmotic adjustment under salt stress. The maintenance of osmotic potential might depend on the accumulation and transport of Na⁺/K⁺ in seeds soaking with GABA in response to salt stress.

GABA exhibits the important function of ROS scavenging in plants [17]. It has been proved that exogenous GABA could significantly improve multiple antioxidant enzyme activities (GPX, SOD, POD, CAT, APX and GR) in rice (Oryza sativa) [35], black pepper (Piper nigrum) seedlings [18], and perennial ryegrass (Lolium perenne) [36], thereby effectively alleviating drought- or heat-caused oxidative damage. Exogenous GABA has also been shown to reduce the low light stress damage through regulating the antioxidant defense system [15]. More importantly, seed soaking with exogenous GABA significantly improved SOD, POD, and CAT activities in roots and leaves of tomato seedlings associated with declines in the production of ROS and oxidative damage under NaCl stress [21]. In the current study, we found that exogenous GABA significantly improved the activities of SOD, POD, CAT, APX, and MDHR in seedlings of white clover under salt stress. Those GABA-activated antioxidant enzymes could play key roles in scavenging ROS such as H₂O₂ and O₂⁻ and reducing membrane lipid peroxidation during seeds germination under salt stress. The study of Shi et al. also found that exogenous GABA could inhibit key genes expression of H₂O₂ synthesis to reduce the accumulation of H₂O₂ in Caragana intermedia roots, suggesting that GABA might act as a signal molecule to regulate the gene expression in response to salt stress [14]. Our findings also suggested that GABA mediated antioxidant defense in white clover during seeds germination at the molecular level through up-regulating multiple genes (Cu/ZnSOD, MnSOD, FeSOD, GPOX, CAT, APX, MDHAR, GST, and GPX) encoding antioxidant enzymes in response to salt stress.

Dehydrins (late embryogenesis abundant protein) play fundamental roles in plant adaptation to abiotic stress. There are almost no dehydrins in the normal vegetative tissue, but dehydrins largely accumulate during seeds germination or under salt, dehydration, freezing, and heat stress in vegetative tissues [37,38]. A large number of transgenic studies revealed positive effects of dehydrins accumulation or the expression of genes encoding dehydrins on stress tolerance in different plant species [37]. The study of Ruibal et al. found that specific dehydrins (PpDHNA, PpDHNB, DHNA and DHNB) could improve salt and drought tolerance of Physcomitrella patens [39]. The study of Hundertmark et al. found that different types of dehydrins (LEA14, XERO1 and RAB18) could protect seeds against deterioration during low moisture storage and increase seeds germination of Arabidopsis thaliana under salt stress [40]. It has been proved that exogenous plant growth regulators such as ABA, cytokinin, PAs, and proline can induce dehydrins accumulation or the expression of genes encoding dehydrins associated with the improvement of stress tolerance in various plants species including white clover [41–43]. The study of Li et al. has found that Spm could significantly promote the accumulation of dehydrins in white clover under osmotic stress, thereby improving the drought tolerance of white clover [44]. In the current study, we found that exogenous GABA significantly increased the accumulation of dehydrins (65 kDa) and expression levels of four genes encoding dehydrins (SK2, Y2K, Y2SK, and dehydrin b) during seeds germination under salt stress, indicating GABA-regulated seeds germination and stress tolerance were closely related to dehydrins accumulation in white clover in response to salt stress.
4. Materials and Methods

4.1. Plant Materials and Treatments

Seeds of white clover (cv. Haifa) were surface-sterilized for 5 min in 0.1% HgCl and then rinsed four times with distilled water (ddH$_2$O). For soaking pretreatment, one set of seeds was soaked in ddH$_2$O for 3 h as control and another set of seeds was soaked in ddH$_2$O for 1 h and then soaked in different concentrations of GABA for 2 h at 20 °C, respectively. The soaked seeds were then germinated in petri dishes (a diameter of 90 mm) with four sheets of filter paper containing 10 mL of 0 or 100 mg·L$^{-1}$ NaCl. Each treatment was replicated six times (50 seeds for each duplicate). The petri dishes were kept in a growth chamber programmed at average day/night temperature of 23/19 °C, 75% relative humidity, and 700 µmol·m$^{-2}$·s$^{-1}$ photosynthetic photon flux density for 7 days. The ddH$_2$O was added in each petri dish every day until the weight of each Petri dish reached its initial weight. Seeds were sampled at 7 d of germination for biochemical and physiological measurements.

4.2. Determination of Seed Germination Characteristics, Root Viability and Endogenous GABA Content

GV was evaluated after 3 d of germination, and GP was evaluated after 7 days of germination. GI and MGT were calculated based on the following formula:

$$GI = \sum \frac{G_t}{T_t}$$

(1)

where $G_t$ is the number of the germinated seeds in the $t$ day; $T_t$ is the time corresponding to $G_t$:

$$MGT = \frac{\sum Ti \times Ni}{\sum Ni}$$

(2)

where $Ni$ is the number of the new germination seeds in times of $Ti$, respectively [10].

After 7 d of germination, RL, SL, shoot-root ratio, FW, DW, and VI were calculated. VI was measured based on the formula $VI = FW \times GI$ [29]. For root viability, fresh roots (0.1 g) were randomly sampled, and then the fresh roots, 0.4% TTC (1 mL), and 0.0667 M phosphate buffer (1 mL, pH 7.4) were added to the pellet and incubated at 37 °C for 1 h in the dark. The sulfuric acid (1 M, 2 mL) was added in order to stop reaction. After moving roots into a new pellet, methanol (5 mL) was added and roots were incubated at 40 °C for 7 h. The absorbance of the supernatant was measured at 485 nm [45].

For the determination of endogenous GABA content, the method of enzyme linked immunosorbent assay (ELISA) was used. The assay kit was purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd., China. Briefly, 0.1 g of seedlings were ground in 1 mL of 0.1 M PBS (pH 7.4) and then centrifuged at 4 °C for 15 min. The supernatant was used for measurement of endogenous GABA content. The procedure was carried out according to the specifications and the absorbance was read at 450 nm after adding stop solution within 15 min on a microplate reader (Synergy HTX, Bio Tek, Winooski, VT USA).

4.3. Determination of Starch Metabolism, Amino Acids, and Osmotic Potential

For water soluble carbohydrate quantification, the procedure was conducted following the method of Fu and Dernoeden [46]. Seedlings (0.5 g) were collected and dried in an oven. Dry tissue (0.05 g) was placed in 10 mL centrifuge tube, and 6 mL ethanol (80%) was added. The mixture was extracted in the water bath at 80 °C for 30 min and then centrifuged at 12,000 g for 10 min. The supernatant was used to measure the content of water-soluble carbohydrate (WSC) [44], and the residue was obtained for starch content analysis [47]. The activities of amylase enzymes were measured by using the method of Tarrago and Kishorekumar et al. [48,49]. Seedlings (0.1 g) were ground with distilled water (8 mL) at 4 °C. The extract was centrifuged at 12,000 g for 25 min at 4 °C. The supernatant was used for estimating α- and β-amylase activities. The 3 mL of supernatant mixed with 3 mL of CaCl$_2$ (3 mM) and
incubated at 70 °C for 5 min. The reaction mixture (0.1 mM citrate buffer, 2% soluble starch solution, and 0.7 mL hot enzyme extract) was incubated at 30 °C for 6 min and then the mixture was heated for 5 min at 50 °C. The α-amylase activity was estimated spectrophotometrically at 540 nm. After inactivating α-amylase at pH 3.4, the β-amylase activity was determined. Reaction solution (2 mL of 0.1 mM citrate buffer, 2% soluble starch, and 0.7 mL EDTA treated enzyme extract) was incubated at 30 °C for 5 min after the addition of starch. The β-amylase activity was then assayed as same as α-amylase.

OP in seedlings was measured according to the method of Blum [50]. Collected seedlings were immediately frozen in liquid nitrogen for 10 min. Seedlings were thawed for 25 min at 4 °C, and then pressed the cell sap for determination of the osmolarity (c) using a sampling chamber of osmometer (Wescor, Logan, UT, USA). The OP was converted based on the formula: MPa = −c \times 2.58 \times 10^{-3}.

Free amino acid content was estimated by the spectrophotometric method using a microplate reader (Synergy HTX, Bio Tek, USA). The assay kit was purchased from Suzhou Comin Biotechnology Co., Ltd., China. Free proline content in seedlings was estimated according to Bates et al. method [51]. 0.1 g of the seedlings sample were weighed and homogenized into a fine paste in 10 mL of 35% sulphosalicylic acid. The homogenate was centrifuged for 10 min. 2 mL of supernatant was mixed with 2 mL of glacial acetic acid and 2 mL of acid ninhydrin solution. The mixture was heated on a water bath for 1 h and the reaction was terminated by keeping in the ice-cold conditions. 4 mL of toluene was added, and the absorbance was read at 520 nm.

4.4. Determination of Antioxidant Enzyme Activities and Oxidative Damage

To analyze antioxidant enzyme activities, fresh seedlings (0.2 g) were ground with 50 mM cold phosphate buffer (4 mL, pH 7.8) containing 1% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 12,000 g for 30 min at 4 °C. The supernatant was used for assays of antioxidant enzyme activities and MDA content. The SOD activity was measured by recording the rate of p-nitroblue tetrazolium chloride reduction of the absorbance at 560 nm [52]. The activity of CAT, GPOX, APX, MDHR, DHAR and GR was determined by following the changes in absorbance at 240, 470, 290, 340, 265 and 340 nm, respectively [53]. Protein content was determined using Bradford’s method [54]. The content of MDA was measured using the method of Dhindsa et al. [55]. Briefly, enzyme extract (0.5 mL) and reaction solution (1 mL) containing 20% (w/v) trichloroacetic acid and 0.5% (w/v) thiobarbituric acid were added to the pellet. The mixture was heated in a water bath at 95 °C for 15 min, and then cooled quickly in an ice-water bath. The homogenate was centrifuged at 8000 g for 10 min. The absorbance of the supernatant was measured at 532, 600 and 450 nm.

The formation rate of O$_2^·−$ was measured using the sulfanilamide method and the absorbance was measured at 530 nm [56]. H$_2$O$_2$ was assayed according to the method of potassium iodide. The oxidation product was measured at 390 nm [57]. For electrolyte leakage (EL), fresh seedlings (0.1 g) were immersed in the centrifuge tube with deionized water (15 mL). The tubes were shaken for 24 h on a shaker table. The conductivity of the solution ($C_{\text{initial}}$) was measured using a conductivity meter (DDS-307A, Shanghai Precision and. Scientific Instrument Co., Ltd., Shanghai, China). Seedlings were then killed by autoclaving at 140 °C for 30 min. The conductivity of killed tissues ($C_{\text{max}}$) was measured. Relative EL was calculated as the percentage of $C_{\text{initial}}$ over $C_{\text{max}}$ [58].

4.5. Determination of Na$^+$/K$^+$ Content and Western Blot Analysis

For Na$^+$/K$^+$ content, seedlings (7 day) were dried at 105 °C for 2 h and then maintained at 75 °C for 72 h. Dry tissue (0.3 g) was added in10 mL of concentrated sulfuric acid, and the mixture was put in graphite digestion instrument (Hanon-SH220N/SH220F, Hanon Subsidiary Company, Shanghai, China). The sample was dissolved completely, and the sample was measured by a flame photometer (Inesa-FP6413, INESA, Shanghai, China). For the analysis of western blot, soluble proteins were extracted from 0.5 g seedlings in ice cold 100 mM Tris-HCl buffer (pH 8.0) and then centrifuged at 12,000 g for 10 min (4 °C). The supernatant was collected and boiled for 10 min. After recentrifuging at
12,000 g, the sediment (an equal amount of 30 µg proteins) was used for determination of dehydrins. The Bio-Rad mini protean transblotter was used for transferring SDS-PAGE (12%) to PVDF membranes. After 2 h of transference at 4 °C and 65 V, the membranes were blocked in TRIS-buffered saline for 1 h [59,60]. When the TRIS-buffered saline was removed, the membranes were washed briefly in TTBS for 3 times each for 5 min. The washed membranes were incubated in rabbit anti-dehydrins dilution (1:1000) as the second antibody for 1 h. After washing in TTBS for 20 min, the dehydrins bands were detected by using the TMB reagent kit (Sigma, Kawasaki, Japan) [61].

4.6. Genes Expression Analysis

Transcript levels of genes were performed using a real-time quantitative polymerase chain reaction (qRT-PCR). For total RNA, the 0.1 g of fresh seedlings was extracted by using RNeasy Mini Kit (Qiagen) according to instructions. A revert Aid First Stand cDNA Synthesis Kit (Fermentas) was used for reverse-transcribing RNA to cDNA. The cDNA was subjected to qPCR using primers of antioxidant enzyme genes (Cu/ZnSOD, FeSOD, MnSOD, CAT, GPOX, APX, MDHR, DHR, GPX, CytGR, GST) [29], dehydrin genes (Y2SK, Y2K, SK2) [60], and Na+/K+ transporter genes (VP1, HKT1, HKT8, SKOR, HAL2, H+−ATPase, SOS1, NHX6) (Table 3) [22]. Transcript level of each gene was measured using an iCyclerIQqRT-PCR detection system with SYBR Green Supermix (Bio-Rad). Four biological replicates with independent cDNA preparations were tested in this study. The conditions of the PCR protocol for all genes were as follows: 5 min at 94 °C and denaturation at 95 °C for 30 s (40 repeats), annealing at 57–66 °C (Table 3) for 30 s and extension at 72 °C for 30 s. At the end of PCR cycle, the transcript level of all genes was calculated according to the formula 2−ΔΔCt described by Xia et al. [62].

| Targetgene | Accession No. | Forward Primer (5′−3′) | Reverse Primer (5′−3′) | Tm (°C) |
|------------|---------------|------------------------|------------------------|---------|
| Cu/ZnSOD   | JQ321597.1    | AACTGTGTACCACGAGGACTTC| AGACTAACAGGCTAAACACG  | 58      |
| FeSOD      | KP202173      | ACAGGATTTCGAGGTTACGACG| GCCGAGGACTACATGTCGACT | 58      |
| MnSOD      | JQ321598.1    | TAAGGGAACTCCGGGAAACT  | CCGAGACACACGTCAACGAC  | 66      |
| CAT        | JQ321596.1    | CAGGACGAGCAGAATTGAGCC| AGACGCTGACACAGGACAGA  | 58      |
| GPOX       | JQ321601.1    | CACCTGAAAAAGTTTTTTGGC| AACAGGTCCTGTCCTGACC   | 64      |
| APX        | JQ321599.1    | AAAAAATATACCAACACCAAA| ACCACTCTTGGGAAACACTG  | 58      |
| MDHR       | KP202172      | CGAATGGCTTAAAGCCTACTG| CAGAGAAGAACTACACGAC   | 64      |
| DHR        | KP202171      | TGCTTACCCCCACCATAT   | TCTTACCAAGAATCTTACG   | 58      |
| GPX        | JQ321604.1    | ATGGCCCTTGGAGCGGTTGAATAC| CCTTTAAGACCAATCTTACG | 58      |
| CytGR      | JQ321602.1    | TAAACTTCACTCCCTTTTCATG| CATAAATTTGTTGGAGACGAC | 58      |
| GST        | JQ321603.1    | TGCTTACCAAGGCACTAACAC| AGACGCTAGACACGACAGATC | 64      |
| SK2        | GU443960.1    | TGGACACGAGTAAACAGACTGGA| TCAGGCTTGGAACTGACCTTGC | 58      |
| Y2K        | JF748410.1    | AGCCACACCCAAAGCTTCTACAA| TGGAGGATGAGGAGTACG | 60      |
| Y2SK       | GU443965.1    | GTGCAAGTAGACATGCTTGGT| CCAAATCTTACCACTGAGCTAGC | 58      |
| dehydrin b | GI443960.1    | TCCAGTACATGCCACCTGTTG| CCAACCAACACCTTCTCGTCA | 58      |
| VP1        | MF403564      | GTTACCACTGACAACTTGGC| AGACGCAAGGACACAAACG   | 60      |
| HKT1       | MF403565      | TGGCAACACCCGAGAAACG | ATCCGAAACCTACACCCATAA | 57      |
| HKT8       | MF403566      | TCTAACCCGGAGAAAGATC | CGATCGAGAGAATACGCGGT | 57      |
| SKOR       | MF403567      | GTCACTTGGCTTGGCAGCTTGGT| CGCCCTTATGGTACACG | 61      |
| HAL2       | MF403568      | TGGTCAGCGTCTGAGGCCC  | TGCCGACTCTCCAGACCTAT | 60      |
| H+−ATPase  | MF403569      | CTTAGTACTGCTGCTGCTTGGC| AATTGAGAAGGACACACCCCTA | 60      |
| SOS1       | MF403570      | TGGTCAGCTTTGAAGTGCAATAC| TCAACAGCAATCCAGAAGCG | 57      |

4.7. Statistical Analysis

The data was analyzed by using SPSS 20 (IBM, Armonk, NY, USA). The significant relationships among the treatments are tested based on differences between means at p ≤ 0.05.

5. Conclusions

Seeds priming with the appropriate concentration of GABA could be an effective technique to alleviate salt-caused inhibition of seeds germination. During germination, seeds soaking with GABA accelerated starch metabolism and utilization of soluble sugar and amino acids contributing to the maintenance of better growth than seeds soaking with H2O under salt stress. In response to salt stress,
GABA-induced increases in Na$^+$/K$^+$ accumulation and transportation could play important roles in osmotic adjustment through offsetting the overconsumption of organic osmolytes during seeds germination. GABA could also increase antioxidant enzyme activities and genes transcript levels associated with better maintenance of cell membrane stability in seedlings of white clover under salt stress. In addition, GABA further increased the accumulation of dehydrins in salt-stressed seedlings, which could be another important regulatory mechanism of salt tolerance in white clover. Current findings provide new evidence for better understanding of GABA-regulated salt tolerance during seeds germination.

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**References**

1. Munns, R. Comparative physiology of salt and water stress. *Plant Cell Environ.* 2002, 25, 239–250. [CrossRef] [PubMed]
2. Ashraf, M. Effect of sodium chloride on water relations and some organic osmotica in arid zone plant species *Melilotus indica* (L.) All. *Tropenlandwirt* 1993, 94, 95–102.
3. Al-Karaki, G. Barley response to salt stress at varied levels of phosphorus. *J. Plant Nutr.* 1997, 20, 1635–1643. [CrossRef]
4. Bybordi, A.; Tabatabaei, S.J.; Ahmedov, A. Effect of salinity on the growth and peroxidase and iaa oxidase activities in canola. *J. Food Agric. Environ.* 2010, 8, 109–112.
5. Bybordi, A.; Tabatabaei, S.J.; Ahmedov, A. Effects of salinity on fatty acid composition of canola (*Brassica napus* L.). *J. Food Agric. Environ.* 2010, 8, 113–115.
6. Woodrow, P.; Ciarmiello, L.F.; Annunziata, M.G.; Pacífico, S.; Iannuzzzi, F.; Mirto, A.; D’Amelia, L.; Dell’Aversana, E.; Piccolella, S.; Fuggi, A.; et al. Durum wheat seedling responses to simultaneous high light and salinity involve a fine reconfiguration of amino acids and carbohydrate metabolism. *Physiol. Plant.* 2016, 159, 290–312. [CrossRef] [PubMed]
7. Liu, K.; Xu, S.; Xuan, W.; Ling, T.; Cao, Z.; Huang, B.; Sun, Y.; Fang, L.; Liu, Z.; Zhao, N. Carbon monoxide counteracts the inhibition of seed germination and alleviates oxidative damage caused by salt stress in oryza sativa. *Plant Sci.* 2007, 172, 544–555. [CrossRef]
8. Cuartero, J.; Bolarin, M.C.; Asins, M.J.; Moreno, V. Increasing salt tolerance in the tomato. *J. Exp. Bot.* 2006, 57, 1045–1058. [CrossRef] [PubMed]
9. Barbagallo, A.; Nicola, A.D.; Missikoff, M. The influence of salt stress on seed germination, growth and yield of canola cultivars. *Notulae Bot. Horti Agrobotanici Cluj-Napoca.* 2010, 38, 128–133.
10. Zhang, S.; Hu, J.; Zhang, Y.; Xie, X.J.; Knapp, A. Seed priming with brassinolide improves lucerne (*Medicago sativa* L.) seed germination and seedling growth in relation to physiological changes under salinity stress. *Aust. J. Agric. Res.* 2007, 58, 811–815. [CrossRef]
11. Duan, D.Y.; Li, W.Q.; Liu, X.J.; Ouyang, H.; An, P.; Duan, D.Y.; Liu, W.Q. Seed germination and seedling growth of saueda salsa under salt stress. *Ann. Bot. Fenn.* 2007, 44, 161–169.
12. Kinnersley, A.M.; Turano, F.J. Gamma aminobutyric acid (GABA) and plant responses to stress. *Crit. Rev. Plant Sci.* 2000, 19, 479–509. [CrossRef]
13. Shelp, B.J.; Bown, A.W.; Mclean, M.D. Metabolism and function of γ-aminobutyric acid. *Trends Plant Sci.* 1999, 4, 446–452. [CrossRef]
14. Shi, S.Q.; Zheng, S.; Jiang, Z.P.; Qi, L.W.; Sun, X.M.; Li, C.X.; Liu, J.F.; Xiao, W.F.; Zhang, S.G. Effects of exogenous GABA on gene expression of caragana intermedia roots under NaCl stress: Regulatory roles for H$_2$O$_2$ and ethylene production. *Plant Cell Environ.* 2010, 33, 149–162. [CrossRef] [PubMed]
15. Li, Y.; Fan, Y.; Ma, Y.; Zhang, Z.; Yue, H.; Wang, L.; Li, J.; Jiao, Y. Effects of exogenous γ-aminobutyric acid (GABA) on photosynthesis and antioxidant system in pepper (Capsicum annuum L.) seedlings under low light stress. *J. Plant Growth Regul.* 2017, 36, 1–14. [CrossRef] [PubMed]
16. Barbosa, J.M.; Singh, N.K.; Cherry, J.H.; Locy, R.D. Nitrate uptake and utilization is modulated by exogenous γ-aminobutyric acid in arabidopsis thaliana seedlings. *Plant Physiol. Biochem.* 2010, 48, 443–450. [CrossRef] [PubMed]
17. Carillo, P. GABA shunt in durum wheat. *Front. Plant Sci.* 2018, 9, 100. [CrossRef] [PubMed]
18. Vijayakumari, K.; Puthur, J.T. γ-Aminobutyric acid (GABA) priming enhances the osmotic stress tolerance in *piper nigrum* linn. plants subjected to PEG-induced stress. *Plant Growth Regul.* 2015, 78, 1–11. [CrossRef]
19. Yu, C.; Zeng, L.; Sheng, K.; Chen, F.; Zhou, T.; Zheng, X.; Yu. T. γ-Aminobutyric acid induces resistance against penicillium expansum by priming of defence responses in pear fruit. *Food Chem.* 2014, 159, 29–37. [CrossRef] [PubMed]
20. Malekzadeh, P.; Khara, J.; Heydari, R. Alleviating effects of exogenous gamma-aminobutric acid on tomato seedling under chilling stress. *Physiol. Mol. Biol. Plants* 2014, 20, 133–137. [CrossRef] [PubMed]
21. Luo, H.Y.; Yang, L.W.; Gao, H.B.; Xiao-Lei, W.U.; Liu, H.H. Physiological mechanism of GABA soaking to tomato seed germination and seedling development under NaCl stress. *Acta Bot. Boreal.-Occident. Sin.* 2011, 31, 2235–2242.
22. Li, Z.; Peng, D.D.; Zhang, X.Q.; Peng, Y.; Chen, M.; Ma, X.; Huang, L.K.; Yan, Y.H. Na+ induces the tolerance to water stress in white clover associated with osmotic adjustment and aquaporins-mediated water transport and balance in root and leaf. *Environ. Exp. Bot.* 2017, 144, 1271–1279. [CrossRef]
23. Khalid, M.; Bilal, M.; Hassani, D.; Hmn, I.; Wang, H.; Huang, D. Mitigation of salt stress in white clover (*Trifolium repens*) by azospirillum brasilense and its inoculation effect. *Bot. Stud.* 2017, 58, 1–7. [CrossRef] [PubMed]
24. Zadeh, H.M.; Naeini, M.S.B. Effects of salinity stress on the morphology and yield of two cultivars of canola (*Brassica napus* L.). *J. Agron.* 2007, 6, 409–414.
25. Hakim, M.A.; Juraimi, A.S.; Hanafi, M.M.; Ali, E.; Ismail, M.R.; Selamat, A.; Karim, S.M. Effect of salt stress on morpha-physiology, vegetative growth and yield of rice. *J. Environ. Biol.* 2014, 35, 317–326. [PubMed]
26. Yacoubi, R.; Job, C.; Belghazi, M.; Chaibi, W.; Job, D. Proteomic analysis of the enhancement of seed vigour in osmoprimed alfalfa seeds germinated under salinity stress. *Seed Sci. Res.* 2013, 23, 99–110. [CrossRef]
27. Wang, Y.; Gu, W.; Yao, M.; Xie, T.; Li, L.; Jing, L.; Shi, W. γ-Aminobutyric acid imparts partial protection from salt stress injury to maize seedlings by improving photosynthesis and upregulating osmoprotectants and antioxidants. *Sci. Rep.* 2017, 7, 43609. [CrossRef] [PubMed]
28. Kim, S.K.; Son, T.K.; Park, S.Y.; Lee, I.J.; Lee, B.H.; Kim, H.Y.; Lee, S.C. Influences of gibberellin and auxin on endogenous plant hormone and starch mobilization during rice seed germination under salt stress. *J. Environ. Biol.* 2006, 27, 181–186.
29. Li, Z.; Peng, Y.; Zhang, X.Q.; Ma, X.; Hang, L.K.; Yan, Y.H. Exogenous spermidine improves seed germination of white clover under water stress via involvement in starch metabolism, antioxidant defenses and relevant gene expression. *Molecules* 2014, 19, 18003–18024. [CrossRef] [PubMed]
30. Ghoulam, C.; Foursy, A.; Fares, K. Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. *Environ. Exp. Bot.* 2002, 47, 39–50. [CrossRef]
31. Saijo, T.; Matsukura, C. Effect of Salt Stress on the Growth and Fruit Quality of Tomato Plants. In *Abiotic Stress Biology in Horticultural Plants*; Kanayama, Y., Kochetov, A., Eds.; Springer: Tokyo, Japan, 2015; pp. 3–16.
32. Zhu, J.K. Regulation of ion homeostasis under salt stress. *Curr. Opin. Plant Biol.* 2003, 6, 441–445. [CrossRef]
33. Shabala, S. Learning from halophytes: Physiological basis and strategies to improve abiotic stress tolerance in crops. *Ann. Bot.* 2013, 112, 1209–1221. [CrossRef] [PubMed]
34. Annunziata, M.G.; Ciarmiello, L.F.; Woodrow, P.; Maximova, E.; Fuggi, A.; Carillo, P. Durum wheat roots adapt to salinity remodeling the cellular content of nitrogen metabolites and sucrose. *Front. Plant Sci.* 2016, 7, 2035. [CrossRef] [PubMed]
35. Nayyar, H.; Kaur, R.; Kaur, S.; Singh, R. γ-Aminobutyric acid (GABA) imparts partial protection from heat stress injury to rice seedlings by improving leaf turgor and upregulating osmoprotectants and antioxidants. *J. Plant Growth Regul.* 2014, 33, 408–419. [CrossRef]
36. Krishnan, S.; Laskowski, K.; Shukla, V.; Merewitz, E.B. Mitigation of drought stress damage by exogenous application of a non-protein amino acid gamma aminobutyric acid on perennial ryegrass. *J. Am. Soc. Hort. Sci.* **2013**, *138*, 358–366.

37. Hanin, M.; Brini, F.; Ebel, C.; Toda, Y.; Takeda, S.; Masmoudi, K. Plant dehydrins and stress tolerance. *Plant Signal. Behav.* **2011**, *6*, 1503–1509. [CrossRef] [PubMed]

38. Marček, T.; Tkalec, M.; Vidaković-Cifrek, Ž.; Ježič, M.; Čurković-Perica, M. Expression of dehydrins, Hsp70, Cu/Zn Sod, and Rubisco in leaves of tobacco (*Nicotiana tabacum* L.) dihaploids under salt stress. *Vitro Cell. Dev. Biol. Plant* **2016**, *52*, 233–240. [CrossRef]

39. Ruibal, C.; Salam, P.; Barbolla, V.; Castro, A.; Bentancor, M.; Borsani, O.; Szabados, L.; Vidal, S. Differential contribution of individual dehydrin genes from *Physcomitrella patens* to salt and osmotic stress tolerance. *Plant Sci.* **2012**, *190*, 89–102. [CrossRef] [PubMed]

40. Hundertmark, M.; Buitink, J.; Leprince, O.; Hincha, D.K. Reduction of seed-specific dehydrins reduces seed longevity in *Arabidopsis thaliana*. *Seed Sci. Res.* **2011**, *21*, 165–173. [CrossRef]

41. Han, B.; Kermode, A.R. Dehydrin-like proteins in castor bean seeds and seedlings are differentially produced in response to ABA and water-deficit-related stresses. *J. Exp. Bot.* **1996**, *47*, 933–939. [CrossRef]

42. Han, B.; Hughes, D.W.; Galau, G.A.; Bewley, J.D.; Kermode, A.R. Changes in late-embryogenesis-abundant (LEA) messenger RNAs and dehydrins during maturation and premature drying of *Ricinus communis* L. seeds. *Planta* **1997**, *201*, 27–35. [CrossRef] [PubMed]

43. Lee, S.P.; Chen, T.H. Molecular cloning of abscisic acid-responsive mRNAs expressed during the induction of freezing tolerance in bromegrass (*Bromus inermis* leys) suspension culture. *Plant Physiol.* **1993**, *101*, 1089–1096. [CrossRef] [PubMed]

44. Li, Z.; Jing, W.; Peng, Y.; Zhang, X.Q.; Ma, X.; Hang, L.K.; Yan, Y.H. Spermine alleviates drought stress in white clover with different resistance by influencing carbohydrate metabolism and dehydrins synthesis. *PLoS ONE* **2015**, *10*, e0120708. [CrossRef] [PubMed]

45. Knievel, D.P. Procedure for estimating ratio of live to dead root dry matter in root core samples. *Crop. J.* **1973**, 13, 124–126. [CrossRef]

46. Fu, J.M.; Dernoeden, P.H. Carbohydrate level, photosynthesis, and respiration in creeping bentgrass as influenced by spring and summer coring. *J. Am. Soc. Hort. Sci.* **2009**, *134*, 41–47.

47. Smith, D. Removing and analyzing total nonstructural carbohydrates from plant tissue. *Jpn. J. Grassl. Sci.* **1969**, *17*, 75–82.

48. Tárrago, J.F.; Nicolás, G. Starch degradation in the cotyledons of germinating lentils. *Plant Physiol.* **1976**, *58*, 618–621. [CrossRef] [PubMed]

49. Kishorekumar, A.; Jaleel, C.A.; Manivannan, P.; Sankar, B.; Sridharan, R.; Panneerselvam, R. Comparative effects of different triazole compounds on growth, photosynthetic pigments and carbohydrate metabolism of solenostemon rotundifolius. *Colloids Surf. B Biointerfaces* **2007**, *60*, 207–212. [CrossRef] [PubMed]

50. Blum, A. Osmotic adjustment and growth of barley genotypes under drought stress. *Crop Sci.* **1989**, *29*, 230–233. [CrossRef]

51. Bates, L.S.; Waldren, R.P.; Teare, I.D. Rapid determination of free proline for water-stress studies. *Plant Soil* **1973**, *39*, 205–207. [CrossRef]

52. Ries, S.K. Superoxide dismutases: I. Occurrence in higher plants. *Plant Physiol.* **1977**, *59*, 309–314.

53. Nakano, Y.; Asada, K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.* **1981**, *22*, 867–880.

54. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]

55. Dhindsa, R.S.; Plumb-Dhindsa, P.; Thorpe, T.A. Leaf senescence: Correlated with increased levels of superoxide dismutase and catalase. *J. Exp. Bot.* **1993**, *47*, 933–939. [CrossRef]

56. Elstner, E.F.; Heupel, A. Inhibition of nitrite formation from hydroxylammonium chloride: A simple assay for superoxide dismutase. *Anal. Biochem.* **1976**, *70*, 616–620. [CrossRef]

57. Velikova, V.; Yordanov, I.; Edreva, A. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. *Plant Sci.* **2000**, *151*, 59–66. [CrossRef]

58. Blum, A.; Ebercon, A. Cell membrane stability as a measure of drought and heat tolerance in wheat. *Crop Sci.* **1981**, *21*, 43–47. [CrossRef]
59. Khedr, A.H.; Abbas, M.A.; Wahid, A.A.; Quick, W.P.; Abogadallah, G.M. Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of *Pancratium maritimum* L. to salt-stress. *J. Exp. Bot.* **2003**, *54*, 2553–2562. [CrossRef] [PubMed]

60. Vaseva, I.; Akiscan, Y.; Demirevska, K.; Anders, I.; Feller, U. Drought stress tolerance of red and white clover–comparative analysis of some chaperonins and dehydrins. *Sci. Hortic.* **2011**, *130*, 653–659. [CrossRef]

61. Close, T.J.; Fenton, R.D.; Moonan, F. A view of plant dehydrins using antibodies specific to the carboxy terminal peptide. *Plant Mol. Biol.* **1993**, *23*, 279–286. [CrossRef] [PubMed]

62. Xia, X.J.; Wang, Y.J.; Zhou, Y.H.; Yuan, T.; Mao, W.H.; Kai, S.; Asami, T.; Chen, Z.X.; Yu, J.Q. Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. *Plant Physiol.* **2009**, *150*, 801–814. [CrossRef] [PubMed]