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Ca²⁺ and aminoguanidine on γ-aminobutyric acid accumulation in germinating soybean under hypoxia–NaCl stress

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ABSTRACT

Gamma-aminobutyric acid (GABA), a nonproteinous amino acid with some benefits on human health, is synthesized by GABA-shunt and the polyamine degradation pathway in plants. The regulation of Ca^{2+} and aminoguanidine on GABA accumulation in germinating soybean (*Glycine max* L.) under hypoxia-NaCl stress was investigated in this study. Exogenous Ca^{2+} increased GABA content significantly by enhancing glutamate decarboxylase gene expression and its activity. Addition of ethylene glycol tetra-acetic acid into the culture solution reduced GABA content greatly due to the inhibition of glutamate decarboxylase activity. Aminoguanidine reduced over 85% of diamine oxidase activity, and 33.28% and 36.35% of GABA content in cotyledon and embryo, respectively. Under hypoxia–NaCl stress, the polyamine degradation pathway contributed 31.61–39.43% of the GABA formation in germinating soybean.

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1. Introduction

Soybean (*Glycine max* L.) is an edible legumes consumed traditionally by Asians and also accepted in Western countries due to its cheap, healthy, and nutritional characters. Apart from dietary protein, carbohydrate, oil, vitamin, and mineral, soybean contains many other functional components (e.g., isoflavone and phosphatidylcholine) [1]. However, its nutritional value is limited by the presence of antinutritional factors such as tannins and enzyme inhibitors [2]. Previous research demonstrated that seed germination could effectively enhance the nutrient value of legumes [3,4]. During seed germination, endogenous enzymes are synthesized or activated to degrade biochemical components such as starch and protein in plant organs, resulting in a significant accumulation of micromolecules including γ-aminobutyric acid (GABA) [5].

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GABA exists widely in prokaryotic and eukaryotic organisms. It is a four-carbon nonproteinous amino acid that acts as a neurotransmitter in the brain and spinal cord of mammals [6]. Development of its functional food can benefit human health. In recent years, GABA-enriched foods have become popular due to its healthy functions for decreasing blood pressure, alleviating pain and anxiety, etc. [7] Seed germination is a simple way for GABA to accumulate. In plant tissues, GABA is biosynthesized through the GABA-shunt [8] and polyamine degradation pathway [9], where glutamate decarboxylase (GAD, EC 4.1.1.15) and diamine oxidase (DAO, EC 1.4.3.6) are the rate-limiting enzymes for GABA formation. In plant cells, GABA is synthesized via the α-decarboxy1ation of glutamate (Glu) in a nonreversible reaction that is cata1ysed by GAD. It is metabolized in the mitochondria to succinic semialdehyde and then succinate by GABA transaminase (EC 2.6.1.19) and succinate semialdehyde dehydrogenase (EC 1.2.1.16), respective1y [8]. This metabolic pathway is called the GABA shunt. GABA can also be formed via the γ-aminobutyraldehyde intermediate from the polyamine degradation reaction where DAO is the key enzyme [10]. A stressful environment such as hypoxia, salt stress, heat or cold shock, drought, and mechanical damage can strongly increase GAD and DAO activities for GABA accumulation [11].

GABA content was greatly enhanced in germinating soybean [12] under hypoxia conditions. Research has shown that hypoxia could lead the acidification of cytoplasm, while the optimum reaction pH of GAD for Glu decarboxylase is 5.5–6.0 [8]. Hence, hypoxia condition was helpful for GABA accumulation. NaCl stress also increased GABA content in germinating soybean [13]. GAD activity can be stimulated by Ca^{2+} [14] because it is a Ca^{2+}/calmodulin-binding protein. In addition, DAO also has a Ca^{2+} binding site [15]. Ethylene glycol-bis-(2-aminoethyl ether)-N,N′-tetra-acetic acid (EGTA) is a metal chelator that may affect GAD and DAO activity by chelating endogenous Ca^{2+}. Aminoguanidine (AG) is a specific inhibitor of DAO can effectively inhibit DAO activity and thus decrease GABA accumulation [13]. Using AG to inhibit DAO activity can investigate the interrelationships between the GABA-shunt and polyamine degradation pathway.

Previous studies mainly focused on either hypoxia or NaCl stress alone. Limited information is available on the comparative studies on an addition of Ca^{2+} and AG for GABA accumulation in germinating seeds under hypoxia-NaCl stress. This scientific research is designed to explicate the influence mechanism of Ca^{2+} and AG on GABA accumulation in germinating soybean, investigate the effects of Ca^{2+} and AG on GAD and DAO activities, and evaluate their gene expression levels under hypoxia–NaCl stress.

2. Materials and methods

2.1. Materials and reagents

Soybean seeds (cultivar *Yunhe*, obtained from Jilin Province of China in 2012) were stored in polyethylene containers at −20°C. Standard samples of GABA (99% in purity), AG, EGTA, and dimethylaminoazobenzene sulfonyl chloride (dabsyl chloride, 99% in purity) were purchased from Sigma Chemical

Co. Ltd. (St Louis, MO, USA). Acetonitrile was high-performance liquid chromatography (HPLC) grade. Other chemicals and reagents were of analytical grade.

2.2. Material treatment and experimental design

Soybean seeds were surface sterilized with 1% of sodium hypochlorite for 30 minutes, washed and steeped with distilled water at 30°C for 4 hours. After germinating for 48 hours in a dark incubator at 30°C, they were placed in cultivating pots with lids (φ 6.0 cm × 18.5 cm) containing 10 mM citrate acid buffer (pH 4.1) by the following treatments based on previous experiments [12,16]. Control: citrate acid buffer as the culture solution (pH 4.1); $CaCl_2$: Control + 6.0mM $CaCl_2$; $CaCl_2$+AG: Control + 6mM $CaCl_2$ + 2.5mM AG; EGTA: Control + 5.0mM EGTA; and EGTA + AG: Control + 5.0mM EGTA +2.5mM AG.

Dissolved oxygen concentration of the culture solution was kept at 5.46 ± 0.02 mg/L by an aerated pump (Yuyao Jintai Meter Ltd., Zhejiang, China) with an airflow rate of 0.9 L/min. After 48 hours of germination under the above treatments, the germinated seeds were washed with distilled water and dried on filter paper, then frozen in liquid nitrogen for further analyses.

2.3. Determination of GABA and Glu

GABA and Glu were extracted and purified according to Bai et al [14]. The residues were dissolved with 2 mL of 1M $NaHCO_3$ (pH 9.0) and centrifuged at 6000 × g for 10 minutes. GABA and Glu were determined by HPLC (Agilent 1200; GMI, Ramsey, MN, USA) with a ZORBAX Eclipse AAA reversed-phase column (3.5 μm), 4.6 mm × 150 mm inner diameter as described by Syu et al [17]. The amino acid solution (1 mL, pH 9.0) was mixed with 1 mL of dabsyl chloride (2 mg/mL, in acetone) and reacting at 67°C for 10 minutes. After that, the reaction was stopped by putting the tubes into an ice bath and then was detected at 425 nm using UV–vis diode-array absorbance detection. The mobile Phase A was acetonitrile and the mobile Phase B was 0.045M CH_3COONa (pH 4), the allowed time of separation of GABA and Glu was within 30 minutes at a constant temperature of 30°C.

2.4. Determination of GAD activity

GAD activity was determined according to Bai et al [14]. One gram of germinated soybean was homogenized on an ice bath with 6 mL of potassium phosphate buffer (70mM, pH 5.8), which contained 2mM β-mercaptoethanol, 2mM ethylene diamine tetraacetic acid (EDTA), and 0.2mM PLP. The homogenate was centrifuged at 10,000 × g for 20 minutes at 4°C, and the supernatant was collected for enzyme assay. The reaction mixture consisted of 200 μL of crude enzyme liquid and 100 μL of substrate (1% of Glu, pH 5.8), incubated at 40°C for 2 hours and then terminated at 90°C for 5 minutes. The centrifugal suspension was filtered through a 0.45-μm membrane filter. The filtrate was analyzed for GABA content. One unit of enzyme activity was defined as the release of 1 μmol of GABA produced per hour at 40°C.

2.5. Determination of DAO activity

DAO activity was determined by the method of Yang et al [18]. Reaction solutions (2.9 mL) contained 2.0 mL of 70mM sodium phosphate buffer (pH 6.5), 0.5 mL of crude enzyme extracts, 0.1 mL of horseradish peroxidase (250 U/mL), and 0.2 mL of 4-aminoantipyrine/N, N-dimethylaniline. The reaction was initiated by adding 0.1 mL of 50mM putrescine (Put). Absorbance at 555 nm was read on a UV-2802 UV—visible spectrophotometer (UNICO, Dayton, NJ, USA). A 0.01 value of change/min in absorbance at 555 nm was regarded as one unit (U) of the enzyme activity.

2.6. Determination of Put

Put was analyzed as described by Xing et al [13]. Tissue (0.5 g) was ground in a mortar with 4 mL 5% (v/v) $HClO_4$. After extraction for 1 hour in an ice bath, the homogenate was centrifuged at $10,000 \times g$ for 20 minutes at $4°C$. Five hundred microliters of the supernatant were mixed with 1 mL 2M NaOH. After the addition of 10 mL benzoylchloride, vortexing for 20 seconds, and incubation for 20 minutes at $37°C$, 2 mL ether was added. After centrifugation at $10,000 \times g$ for 5 minutes at $4°C$, 1 mL of the ether phase was collected, evaporated to a dry state, and redissolved in 100 mL methanol. Benzoyl-polyamines (20 μL) were analyzed using HPLC (G 1314B, Agilent 1200) with a ZORBAX Eclipse AAA reversed-phase column (3.5 μm, 4.6 mm × 150 mm). Methanol:H_2O (64:36, v:v) was used as an isocratic eluting solvent at 0.6 mL/min.

2.7. RNA extraction and reverse-transcription polymerase chain reaction analysis

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from cotyledon and embryo of the germinated soybean. First-strand cDNA was synthesized using a reverse-transcription polymerase chain reaction (RT-PCR) Kit (DR027S; TaKaRa, Dalian, Liaoning, China). For PCR amplification of GAD and DAO genes, names and sequence-specific forward and reverse primers are listed in Table 1. Actin (V00450) was used as the internal control. For determining the expression pattern of each gene, RT-PCR analyses were carried out ($n = 3$). The PCR was conducted as following: $94°C$ for 2 minutes; 32 cycles (GAD and DAO) or 25 cycles (Actin) at $94°C$ for 30 s, $56°C$ (GAD) or $55°C$ (DAO) for 30 seconds or $53°C$ (Actin) for 20 seconds, $72°C$ for 60 seconds; and $72°C$ for 10 minutes. Amplified fragments were obtained and detected by electrophoresis on a 1.5 % (w/v) agarose gel and visualized by ethidium bromide.

2.8. Statistical analysis

Version 9.1.3 of SAS program (SAS Institute Inc., Cary, NC, USA) was used for analysis of variance (ANOVA) to assess the data. Duncan's protected multiple comparisons were used to identify statistical significance at a level of $p < 0.05$.

3. Results and analyses

3.1. Effects of Ca^{2+} and AG on GABA accumulation

Ca^{2+} and AG affected GABA accumulation in both cotyledon and embryo of germinating soybean (Fig. 1). Under hypoxia-NaCl, 6.0mM exogenous $CaCl_2$ increased GABA content in cotyledon and embryo by 30% and 16%, respectively. However, AG combined with $CaCl_2$ decreased GABA content by 33.28% and 36.35% in cotyledon and embryo, respectively, compared with that of adding $CaCl_2$ alone. EGTA reduced GABA accumulation in cotyledon and embryo by 20.38% and 23.44% compared to that of the control. The addition of AG further decreased GABA content in cotyledon and embryo. These results clearly reveal that Ca^{2+} plays a significantly positive role, while AG and EGTA both perform a significantly negative role in GABA accumulation of germinating soybean.

3.2. Effects of Ca^{2+} and AG on Glu and Put content

Exogenous $CaCl_2$ significantly decreased Glu content in cotyledon and embryo by 39.01% and 29.59%, respectively (Fig. 2A), while increased Put content in the embryo (Fig. 2B). Addition of both $CaCl_2$ and AG simultaneously did not significantly change Glu content compared to that of adding $CaCl_2$ alone ($p > 0.05$; Fig. 2A), but it significantly enhanced Put content in cotyledon and embryo ($p < 0.05$; Fig. 2B). After adding EGTA, Glu content in cotyledon and embryo increased by 18.32% and 35.49%, respectively (Fig. 2A), and Put content increased by 162.39% and 26.41% in cotyledon and embryo, respectively (Fig. 2B). EGTA combined with AG treatment raised Put content in cotyledon and embryo by 109.68% and 76.92%, respectively (Fig. 2B). These results indicate that Ca^{2+} enhanced Glu degradation to GABA significantly, while EGTA caused an inhibition of GABA formation from Glu and Put, and

Gene	Primer sequence	Fragment size (bp)	Ta (°C)	Genebank accession no.
GAD	Sense: GAAAGCCTTATGACAAACCC Antisense: CTTGAGAAGTCCTCCCTGAT	815	56	AY323131.1
DAO	Sense: ATGGCTTCCCTACCCTTTCC Antisense: GTTGACTTCCGTCTCGTTTC	831	55	AJ583529.1
Actin[a]	Sense: TGATGGTGTGAGTCACACTGTACC Antisense: GGACAATGGATGGGCCAGACTC	644	53	V00450

Table 1 – Information of primers for reverse-transcription polymerase chain reaction analysis of genes expression.

Ta = annealing temperature.
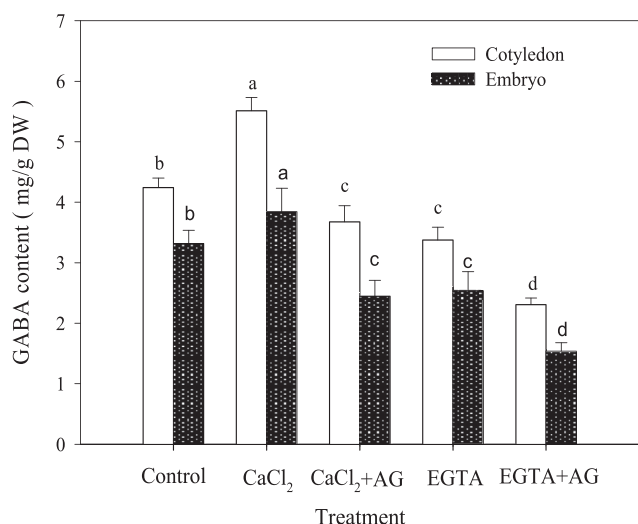[a] Housekeeping gene for internal control.

Fig. 1 – Effects of additives on GABA accumulation in germinating soybean. Different letters above each bar denote significance of Duncan test ($p < 0.05$) and represent statistical results from multiple comparisons of germinating soybean cotyledon or embryo in different treatments. Control = 60mM NaCl; CaCl$_2$ = Control + 6.0mM CaCl$_2$; CaCl$_2$+AG = Control + 6.0mM CaCl$_2$+2.5mM AG; EGTA = Control + 5.0mM EGTA; EGTA+AG = Control + 5.0mM EGTA + 2.5mM AG; DW = dry weight.

AG played a further negative role in increasing GABA content in germinating soybean.

3.3. Effects of Ca^{2+} and AG on GAD and DAO activity

Exogenous CaCl$_2$ improved GAD activity by 29% in the embryo (Fig. 3A), while it had no effect on DAO activity (Fig. 3B). CaCl$_2$ combined with AG did not change GAD activity compared with that of CaCl$_2$ treatment alone ($p > 0.05$; Fig. 3A); instead, it significantly inhibited DAO activity ($p < 0.05$) in cotyledon and embryo by 87.92% and 99.75%, respectively (Fig. 3B). The 5.0mM of EGTA significantly inhibited GAD activity ($p < 0.05$) in

cotyledon and embryo (Fig. 3A), but it did not affect DAO activity (Fig. 3B). When cotreated using EGTA and AG, GAD activity was significantly reduced ($p < 0.05$) in the embryo, but there was no significant alteration in cotyledon ($p > 0.05$) compared to that treated with EGTA alone (Fig. 3A). However, DAO activity in the embryo and cotyledon was inhibited significantly ($p < 0.05$; Fig. 3B). As a result, it was decreased by 93.92% and 99.59%, respectively, in comparison with that treated by EGTA alone. These results indicate that Ca^{2+} is helpful for GAD activity increase in the embryo of germinating soybean; instead, EGTA as a chelating agent of Ca^{2+} can decrease GAD activity. AG significantly inhibited DAO activity during soybean seeds germination.

3.4. Ca^{2+} and AG on GAD and DAO expression

The GAD and DAO genes had different expression levels in germinating soybean (Fig. 4). In cotyledon, exogenous CaCl$_2$ improved GAD (Fig. 4A) and DAO (Fig. 4B) expression by 13% and 171%, respectively. Adding both CaCl$_2$ and AG did not affect the expression of GAD significantly compared to that of adding CaCl$_2$ alone (Fig. 4A), while this inhibited DAO expression by 28.22% (Fig. 4B). Exogenous EGTA improved GAD (Fig. 4A) and DAO (Fig. 4B) expression by 30.64% and 184.12%, respectively. Adding both EGTA and AG (Fig. 4) did not affect the expression level of GAD ($p > 0.05$), but significantly enhanced DAO expression ($p < 0.05$) compared to that of adding EGTA alone (Fig. 4).

The expression levels of GAD and DAO in the embryo of germinating soybean are shown in Fig. 4C and D, respectively. Adding 6.0mM CaCl$_2$ increased the expression of GAD by 64.12% in comparison with that of the control (Fig. 4C). Both AG and CaCl$_2$ improved GAD expression in the embryo. Adding EGTA significantly enhanced GAD expression, but its impact was less efficient than that of adding CaCl$_2$. EGTA and AG further promoted GAD expression in the embryo. However, there were no significant differences ($p > 0.05$) of DAO expression in the embryo among all treatments in the present study (Fig. 4D).

In summary, under hypoxia-NaCl stress, Ca^{2+}, EGTA, and AG induced the GAD expression in cotyledon and embryo of
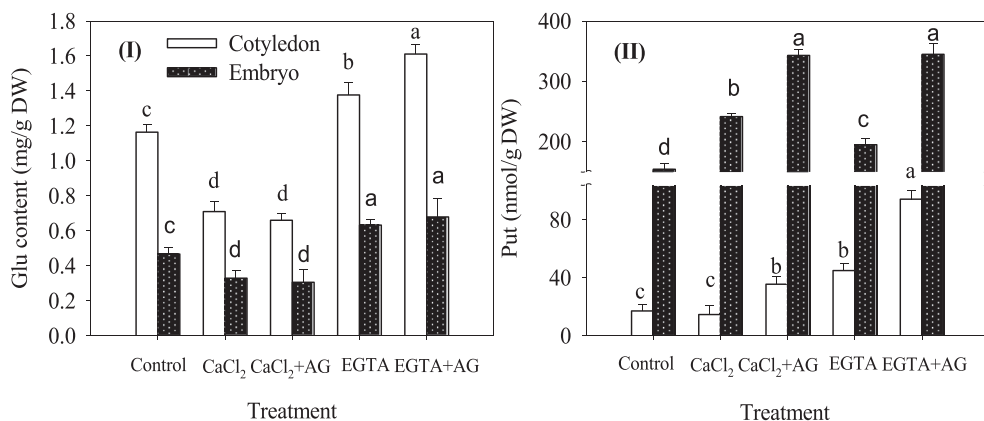

Fig. 2 – Effects of additives on (A) glutamate (Glu) and (B) putrescine (Put) contents in germinating soybean. Treatments and statistics are the same as those in Fig. 1.
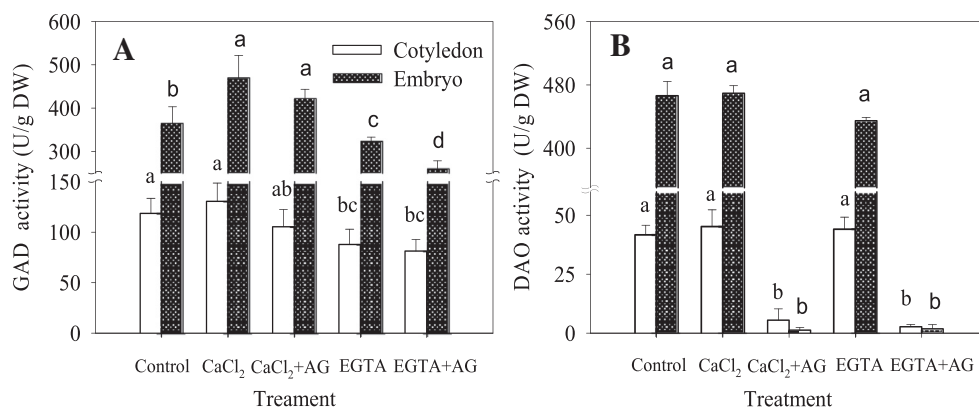
Fig. 3 − Effects of additives on (A) glutamate decarboxylase (GAD) and (B) diamine oxidase (DAO) activities in germinating soybean. Treatments and statistics are the same as those in Fig. 1.

geminating soybean, and only induced *DAO* gene expression in cotyledon while have no significant influence in the embryo.

4.   Discussion

GABA-rich foods are increasingly popular for their functional effects on human health. In plant tissues, the biosynthesis of GABA can be an adaptive response to stress-induced cellular acidosis. Up to now, little attention has been paid to the polyamine degradation pathway in research into GABA accumulation, even though polyamine degradation provides about 39% of GABA formation, increased by DAO, in germinating soybean roots under NaCl stress [13]. In the present study, under hypoxia−NaCl stress, addition of AG in the presence of CaCl$_2$ and EGTA significantly inhibited DAO activity. As a consequence, GABA content was decreased by
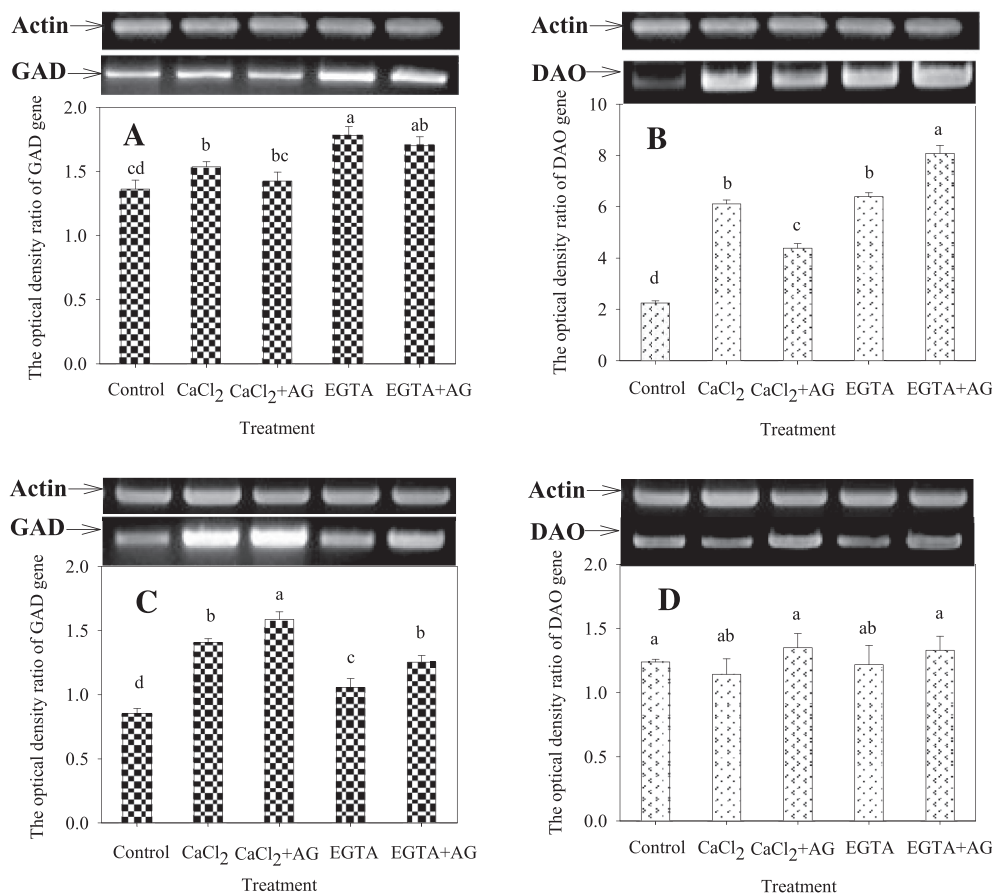

Fig. 4 − (A) glutamate decarboxylase (GAD) and (B) diamine oxidase (DAO) expression in cotyledon; (C) GAD and (D) DAO expression in embryo of germinating soybean. Treatments and statistics are the same as those in Fig. 1.

31.61% and 33.28% in cotyledon and 36.35% and 39.43% in the embryo of germinating soybean, respectively (Fig. 1). Thus, it can be concluded that contribution of polyamine degradation to GABA accumulation varies with not only plant organs but also with the environmental conditions during soybean germination.

Bouché et al [11] demonstrated that a rapid increase in the cytoplasm Ca^{2+} level in plant cells under stress could result in the stimulation of GAD activity and the increase of GABA content. The present results revealed that Ca^{2+} increased GABA content significantly under hypoxia-NaCl of germinating soybean. This effect resulted from the improvement of both GAD activity and its gene expression level, and suggests that addition of Ca^{2+} could not only activate GAD activity, but also regulate its gene expression for biosynthesis of new GAD, which finally contributed to GABA accumulation by enhancing Glu conversion. Interestingly, Ca^{2+} raised Put content in the embryo and promoted *DAO* gene expression in cotyledon, but could not improve DAO activity. The reason might be that, on one hand, the mRNA was not translated completely [19] when the level of *DAO* transcription increased; on the other hand, it also hinted that Ca^{2+} mainly promoted GABA accumulation by activating GAD in GABA shunt. In the present study, GABA contents in cotyledon and embryo reduced significantly when treated with EGTA, a commonly used metal chelator. Soybean GAD is a Ca^{2+} binding protein [19], and its DAO contains Ca^{2+} and Cu^{2+} [20], so their activities are associated with the existence of metal chelators. As a result, the activities of the two enzymes could be subsequently reduced when EGTA is added, resulting in a decrease of GABA formation. When the activity of GAD or DAO was partially inhibited, GABA metabolic pathways were consequently inhibited. Therefore, this could regulate both *GAD* and *DAO* expression to generate new GAD and DAO for GABA biosynthesis in organs. Therefore, EGTA inhibited GABA level by inhibiting GAD and DAO activity in the germinating soybean.

AG is a specific inhibitor of DAO [21]. DAO activity in germinating soybean was inhibited significantly by AG, and blocked the degradation of polyamine in this study. As a result, Put accumulated, but GABA content decreased. The *DAO* gene in soybean roots has high and stable expression, ensuring the normal metabolism of Put in soybean roots [22,23]. Similar to proline, GABA is a good osmotic regulator in plant organs. In this study, *DAO* gene expression was stable in soybean embryos to increase GABA formation. When DAO activity was inhibited, polyamine degradation was blocked, so that GABA formation decreased. In order to minimize the phytophysiological damage to cells, germinating soybean synthesized GABA to regulate biochemical metabolism activities by inducing *GAD* and *DAO* gene expression to synthesize new enzymes.

5.   Conclusion

Under hypoxia—NaCl stress, Ca^{2+} increased GABA content significantly by enhancing GAD activity and upregulating its gene expression. EGTA greatly reduced GABA content due to the inhibition of GAD and DAO activity. And AG reduced

33.28% and 36.35% of GABA content in germinating soybean cotyledon and embryo, respectively, by the inhibition of DAO activity. In other words, about 33.28% and 36.35% of GABA was contributed by the pathway of polyamine degradation in cotyledon and embryo, respectively, under hypoxia-NaCl stress.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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