Polyamine Degradation Pathway Regulating Growth and GABA Accumulation in Germinating Fava Bean under Hypoxia-NaCl Stress

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ABSTRACT

In plants, γ-aminobutyric acid (GABA) is synthesized by polyamine degradation pathway besides GABA shunt. Aminoguanidine (AG) is a specific inhibitor of the key enzyme (diamine oxidase, DAO) for GABA formation in polyamine degradation pathway. In this study, AG was applied to study the functions of polyamine degradation pathway on growth and GABA accumulation in germinating fava bean under hypoxia-NaCl stress. The results showed that 5.0 mmol L^{-1} of AG inhibited DAO activity maximally but not entirely, and inhibited the growth of sprouts simultaneously. Hence, blocking polyamine degradation pathway significantly affected the growth of germinating fava bean. Polyamine degradation pathway provided 26.9 and 29.3% of GABA in cotyledon and embryo, respectively, because DAO activity was not inhibited entirely. Polyamine, especially putrescine (Put), accumulated after polyamine degradation pathway was blocked, indicating that Put was the main substrate of GABA in polyamine degradation pathway.

Keywords: Aminoguanidine, Diamine oxidase, Putrescine, γ-aminobutyric acid.

INTRODUCTION

Fava bean (*Vicia faba* L.) is one of the common legumes rich in dietary proteins, carbohydrates, and other necessary compounds for human diet. Germination not only can modify nutrients and anti-nutrients in fava bean but also increase bioactive substances content or produce some new functional components like γ-aminobutyric acid (GABA) (Li *et al.*, 2010). GABA is a non-protein amino acid with some functional properties for human health such as lowing blood pressure and regulating heart rate (Mody *et al.*, 1994).

GABA is widely present in prokaryotic and eukaryotic organisms. In recent years, GABA-enriched foods have become popular, such as GABA-tea (Syu *et al.*, 2008), GABA-brown rice (Komatsuzaki *et al.*, 2007), GABA-soy bean sprouts (Guo *et al.*, 2012). In plant cells, GABA is synthesized via the α-decarboxy1ation of glutamate (Glu) in an irreversible reaction which is cata1yzed by glutamate decarboxylase (GAD, EC 4.1.1.15) (Bown *et al.*, 1997). This metabolic pathway is called GABA shunt. In addition, GABA can also be formed via γ-aminobutyraldehyde intermediate from polyamine degradation reaction where diamine oxidase (DAO, EC 1.4.3.6) is the key enzyme (Wakte *et al.*, 2011). Researches on GABA accumulation in germinating seeds focus on GABA shunt (Bai *et al.*, 2009; Mae *et al.*, 2012), but little information is available on polyamine degradation pathway (Xing *et al.*, 2007). In the majority of germinating seeds, stressful

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conditions such as hypoxia (Guo *et al.*, 2011), salt stress (Widodo *et al.*, 2009) and drought (Kramer *et al.*, 2010) can strongly increase GABA content. During fava bean germination under non-stress condition, GABA content increased slightly (Yang *et al.*, 2011), but it increased significantly when germinating under hypoxia stress (Yang *et al.*, 2013). At present, few studies on GABA accumulation in germinating fava bean under salt stress are available. Under these stressful conditions, the relationship between GABA shunt and polyamine degradation pathway is still not clear.

Aminoguanidine (AG) is a specific inhibitor of DAO which can block polyamine degradation pathway via the inhibition of DAO activity (Yang *et al.*, 2012). When AG is added during seed germination, polyamine degradation pathway is blocked so as to investigate its functions in plant conveniently. Recently, researchers have used this way and investigated the contribution of polyamine degradation pathway for GABA accumulation in germinating soybean under salt stress (Xing *et al.*, 2007). Yang *et al.* (2011) showed that about 30% of the GABA content in fava bean germinating under non-stress condition was supplied by polyamine degradation pathway. However, under hypoxia-NaCl stress, little information about polyamine degradation pathway on growth and GABA accumulation is available.

In the present study, AG was applied to block polyamine degradation pathway by inhibition of DAO activity. Then, growth and the relationship among GABA accumulation, DAO activity, and precursors of GABA (polyamine) contents of hypoxia-NaCl stressed fava bean sprouts were investigated as a preliminary exploration of the function of polyamine degradation pathway.

MATERIALS AND METHODS

Materials and Reagents

Dried fava beans (Qi Bean 2), harvested in 2011, were supplied by Jiangsu Academy of Agricultural Sciences (Nanjing, China) and stored at -20°C before the experiments. Horseradish peroxidase, putrescine (Put), aminoguanidine (AG), 4-aminoantipyrine and *N*,*N*-dimethylaniline were purchased from Sigma Chemical Co. (St. Louis, USA). All reagents were of analytical grade.

Seed Pretreatment

Dry seeds were surface-sterilized with 10 mL L^{-1} sodium hypochlorite solution for 30 min, thoroughly rinsed with distilled water and steeped in distilled water at 30±1°C for 8 hours. After that, the soaked seeds were put in culturing pallet (25×20×5 cm, L×W×H) with sterilized quartz sand (2 cm thick), covered with fresh-keeping film with eyelets and incubated at 30°C in darkness for 2 days.

AG Concentration Selection

Pretreated fava beans (30 seeds) were placed in cultivated pots with lids (φ 6×18.5 cm) and germinated with 0.0, 2.5, 5.0, 7.5 and 10.0 mmol L^{-1} of AG, respectively, under hypoxia-NaCl stress (culture solution, pH 3.5 (Yang *et al.*, 2013), contained 60 mmol L^{-1} NaCl besides AG, aerated by a pump to ensure final dissolved oxygen concentration was 5.5 mg L^{-1}) for 4 days at 30±1°C in darkness. After that, sprouts were carefully collected and washed with distilled water for indices determination.

AG Treatment

Pretreated fava beans (30 seeds) were placed in cultivated pots with lids (φ 6×18.5 cm) and subjected to hypoxia-NaCl stress and germinating for 4 days, sampling each day and analyzing sprout length, GABA content, DAO activity, and polyamine content. The seed germinating without AG was taken as the control.

Sprout Length Determination

Sprout length was directly measured with a centimeter ruler directly (the 25 fava bean sprouts were measured for one sample).

Determination of GABA Content

Fresh sprouts (1.0 g) were mulled with 5 mL of 7% acetic acid. The purification of GABA was according to Yang *et al.* (2011). The purified supernatant was evaporated (0.1 MPa, 45°C) to volatilize the acetic acid and ethanol. The residues were dissolved with 2 mL of 1.0 mol L^{-1} of $NaHCO_3$ (pH 9) and centrifuged at 8,000×g for 10 minutes. GABA was determined by HPLC (Agilent 1200, USA) with a ZORBAX Eclipse AAA reversed-phase column (4.6×150 mm id, 3.5 μm particle size) as described by Guo *et al.* (2012) The amino acid solution (1.0 mL, pH 9.0) was mixed with 1.0 mL of dabsyl chloride (2.0 mg mL^{-1}, in acetone) and reacted at 67°C for 10 minutes. After that, the reaction was stopped by an ice bath and then was detected at 425 nm using UV–vis diode-array absorbance detection (DAD). The mobile phase A was acetonitrile and the mobile phase B was 45 mmol L^{-1} of CH_3COONa (pH 4.0); the allowed time of separation of GABA was within 30 minutes at a column temperature of 30°C. The elution program was the same as that of Yang *et al.* (2011)

Determination of DAO Activity

DAO activity was determined using the method of Yang *et al.* (2012). Reaction solutions (2.9 mL) contained 2.0 mL of 70 mmol L^{-1} sodium phosphate buffer (pH 6.5), 0.5 mL of crude enzyme extracts, 0.1 mL of horseradish peroxidase (250 U mL^{-1}) and 0.2 mL of 4-aminoantipyrine/N, N-dimethylaniline. The reaction was initiated by adding 0.1 mL of 50 mmol L^{-1} Put. Absorbance at 555 nm was read on UV-2802 UV–visible spectrophotometer (UNICO, USA). A 0.01 value of the changes per minute in absorbance at 555 nm was regarded as one activity unit (U) of the enzyme.

Determination of Protein Content

Protein content of the supernatants was measured by a protein-dye reagent (Coomassie blue G-250) with bovine serum albumin as the standard (Yang *et al.*, 2011).

Polyamines Content Determination

Free polyamines were analyzed by HPLC as described by Xing *et al.* (2007). The HPLC system (Agilent 1200, USA) with a ZORBAX Eclipse AAA reversed-phase column (4.6×150 mm id, 3.5 μm particle size) was used. Methanol:H_2O (64:36, v:v) was used as an isocratic eluting solvent at 0.6 mL/min.

Statistical Analysis

Means and standard deviations (means±SD) were computed for experimental data. Statistical analysis was performed using Fisher's *F*-test at significance level of $P < 0.05$.

RESULTS

Selection of AG Concentration

With the increase of AG concentration, DAO activity of cotyledon and embryo was inhibited significantly ($P < 0.05$) (Table 1), which was 22.2 and 9.7% of the control in cotyledon and embryo, respectively, after the 5.0 mmol L^{-1} AG treatment. However, when AG concentration was above 5.0 mmol L^{-1}, no significant changes in DAO activity were observed, thus, the 5.0 mmol L^{-1} of AG inhibited the DAO activity the furthest. The changing pattern of GABA

Table 1. Effect of AG concentration on DAO activity and GABA content in hypoxia-NaCl stressed fava bean.

Index	Organs	AG concentration (mmol L^{-1})				
		0.0	2.5	5.0	7.5	10.0
DAO activity	Cotyledon	14.64±2.30^a	9.51±1.20^b	5.48±1.50^c	4.14±0.50^c	4.07±0.90^c
(U g^{-1} DW)	Embryo	28.46±3.20^a	5.15±1.10^b	2.75±1.20^c	2.75±0.50^c	2.67±0.30^c
GABA content	Cotyledon	1.72±0.18^a	1.37±0.09^b	1.29±0.06^b	1.28±0.08^b	1.09±0.04^c
(mg g^{-1} DW)	Embryo	8.01±0.50^a	7.52±0.33^b	5.66±0.22^c	5.53±0.25^c	4.83±0.22^d

Values are the means of triplicate analyses. Data was shown as Means±SD. Those with different lower case letters in the same row are significantly different at $P< 0.05$.

content was similar to that of DAO activity but it decreased more slowly.

Sprout Length and Fresh Weight during Germination

Treating with 5.0 mmol L^{-1} of AG, sprout length showed the same trend with the control, but was shorter (Figure 1-A). The decrement of which (Y) with treating time (X) could be expressed as the regression equation of $Y=-0.1929X^2+1.6614X-0.0457$, (0< X≤ 4), $R^2= 0.9969$ (Figure 1-C). It was calculated that sprout length decreased by 31.0% on the average. In addition, AG decreased the fresh weight of sprouts by 12.3% on the average (Figure 1-B). The decrement of fresh weight (Y) with germination time (X) was expressed as the regression equation of $Y=0.8646X^3-6.3036X^2+16.087X+0.1904$, (0< X≤4), $R^2= 0.9877$ (Figure 1-D). These may be attributed to the growth rate of new tissues, which was slowed down after AG treatment, so that the extension of the sprouts was inhibited significantly (Figure 1-A). As a result, the water absorption rate decreased and, simultaneously, the fresh weight decreased (Figure 1-B). This indicates that the eubolism of polyamine degradation pathway is very important for plant growth.

DAO Activity and GABA Accumulation during Germination

Compared to the control, DAO activity in cotyledon and embryo decreased continuously with the treatment time, it was 36.7 and 12.3% of the control in cotyledon (Figure 2-A) and embryo (Figure 2-B), respectively, in 4-day germinating fava bean. The relationship between DAO activity decrement (Y) and treatment time (X) in cotyledon was expressed as the regression equation of $Y=-0.92X^2+6.1089X-0.309$, (0< X≤ 4), $R^2= 0.9853$ (Figure 2-C); and in embryo it was expressed as $Y=-1.8733X^3+10.216X^2-4.3773X-0.1791$, (0< X≤ 4), $R^2= 0.9965$ (Figure 2-D).
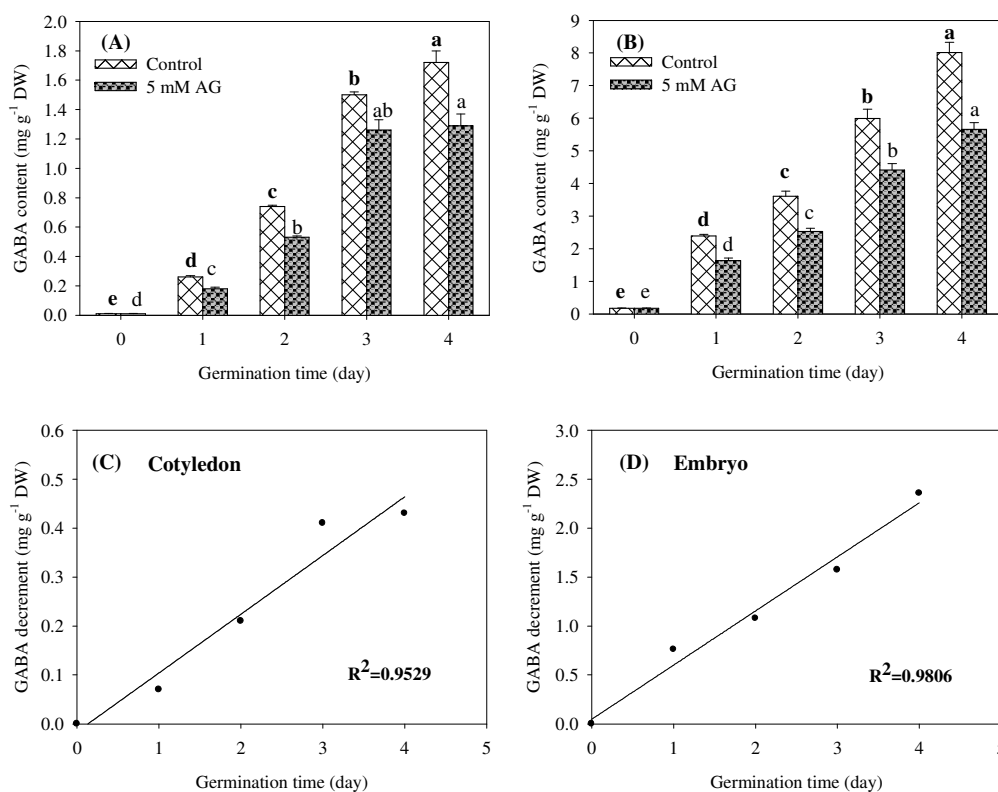
GABA content in cotyledon and embryo increased significantly (P< 0.05) with the germination time. When treating with 5.0 mmol L^{-1} of AG, GABA content decreased linearly compared to that of the control (Figure 3A and 3B). The decrement in cotyledon and embryo with treatment time was expressed as the regression equations of $Y=0.092X+0.078$ and $Y=0.5524X+0.0483$, (0< X≤ 4), respectively [Figure 3, (C and D)]. It was calculated that GABA content in cotyledon and embryo decreased on the average by 26.9 and 29.3%, respectively, via equations.
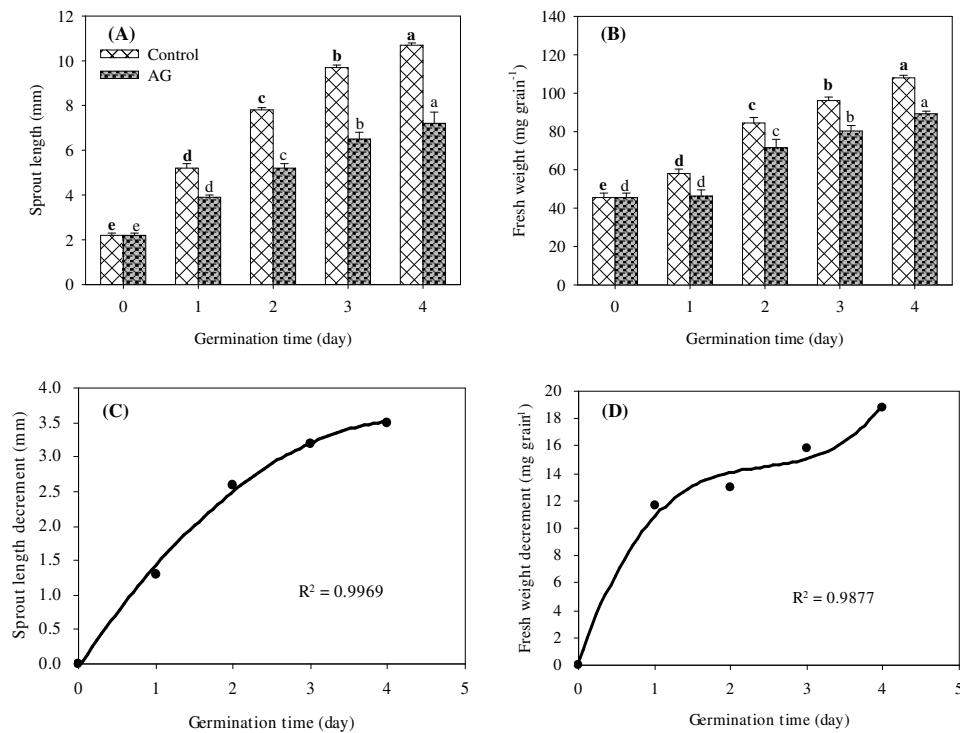
Figure 1. Effects of AG treating time on sprout length (A) and the fresh weight (B) of fava bean sprouts. The decrement of sprout length and fresh weight is shown in (C) and (D), respectively. Values are the means of triplicate analyses. Error bars show the standard deviation. Those with different lower case letters in the same filled pillar are significantly different at *P*< 0.05.
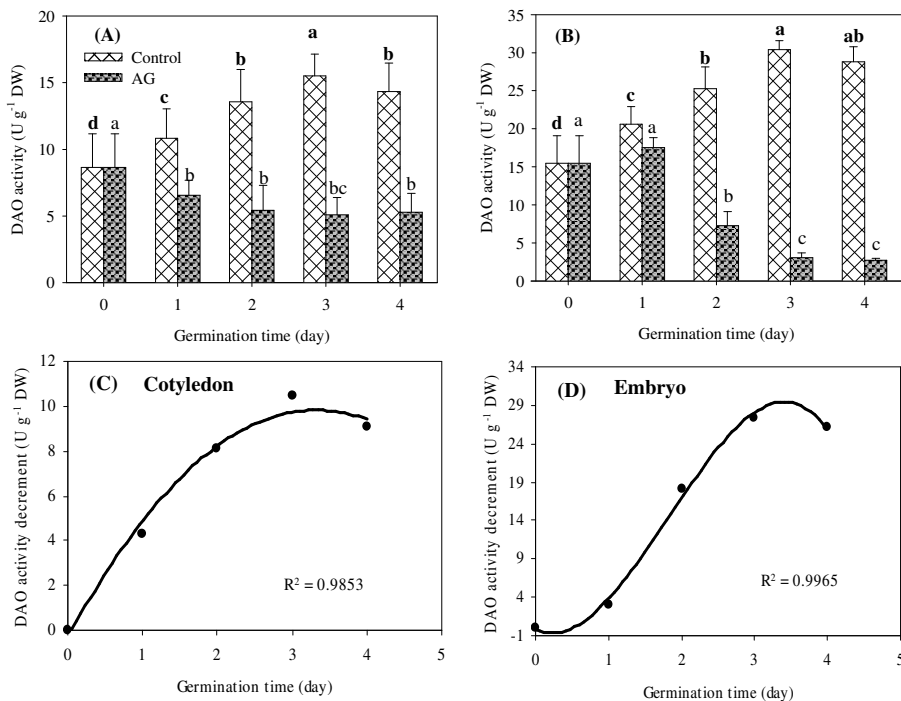
Figure 2. Effects of AG treating time on DAO activity of cotyledon (A) and embryo (B) of fava bean sprouts. The decrement of DAO activity in cotyledon and embryo is shown in (C) and (D), respectively. Values are the means of triplicate analyses. Error bars show the standard deviation. Those with different lower case letters in the same filled pillar are significantly different at *P*< 0.05.
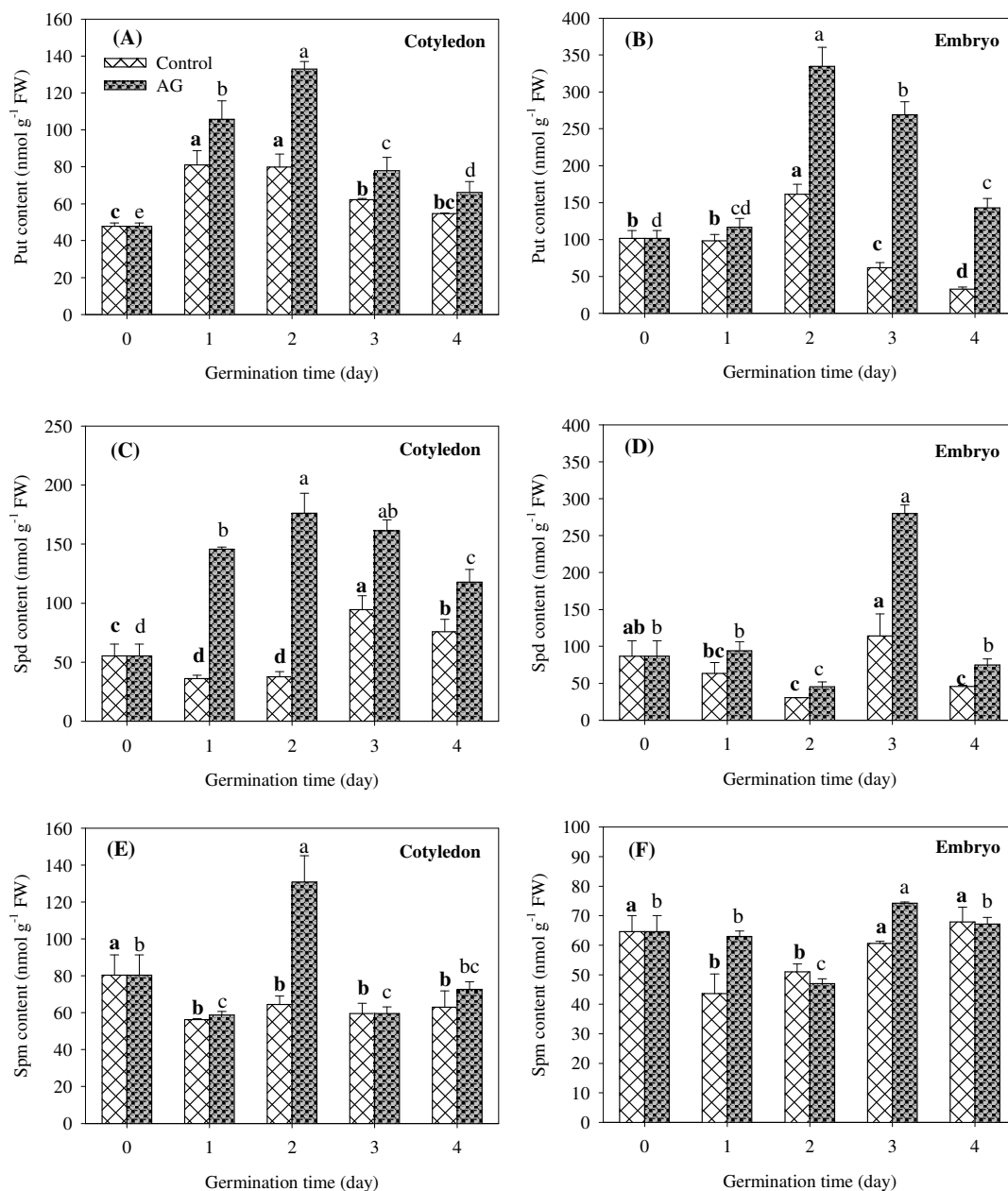
Figure 3. Effects of AG treating time on GABA content in cotyledon (A) and embryo (B) of germinating fava bean. The decrement of GABA content in cotyledon and embryo is shown in (C) and (D), respectively. Values are the means of triplicate analyses. Error bars show the standard deviation. Those with different lower case letters in the same filled pillar are significantly different at $P< 0.05$.

Polyamine Content during Germination

"Put" content in cotyledon and embryo under AG treatment increased firstly and then decreased and were higher than that of the controls, the highest values were observed two days after germination. They were 1.66- and 2.08-fold of the control, respectively. Spd also accumulated under AG treatment and reached the highest value two days and three days after germination in cotyledon (Figure 4-C) and embryo (Figure 4-D), respectively. The changes of Spm in cotyledon and embryo was not regular, but in cotyledon, it was 102.9% higher than that of the control two days after germination (Figure 4-E), while in embryo there was no obvious trend (Figure 4-F).

DISCUSSION

Numerous studies about GABA accumulation are focused on GABA shunt, however, little information is available on polyamine degradation pathway. In legumes, L-arginine content is relatively high and can be converted into polyamines (Vuosku *et al.*, 2006), which are oxidized to GABA further (Alcazar *et al.*, 2010). Hence, polyamine degradation pathway is very important for GABA accumulation in legumes. Xing *et al*. (2007) reported that polyamine degradation pathway supplied about 39% of GABA in soybean seedling root under NaCl stress. Under non-stress condition or hypoxia stress, polyamine degradation pathway provided about 30% of GABA formation in germinating fava bean.

Figure 4. Effects of AG treating time on polyamines content of germinating fava bean. (A); (C), and (E) show the content in cotyledon, (B); (D), and (F) show the content in embryo. Values are the means of triplicate analyses. Error bars show the standard deviation. Those with different lower case letters in the same filled pillar are significantly different at $P < 0.05$.

DAO is a key enzyme for GABA biosynthesis by polyamine degradation pathway (Matilla *et al.*, 2002), while AG is a specific inhibitor of DAO and could decrease GABA accumulation by the inhibition of DAO activity (Yang *et al.*, 2012). In the present study, the 5.0 mmol L^{-1} of AG not only inhibited DAO activity but also inhibited the growth of fava bean sprouts. This indicates that polyamine

degradation pathway is very important for plant development.

Sometimes AG could not block the polyamine degradation pathway completely, although it is a specific inhibitor of DAO. In the present research, 5.0 mmol L^{-1} of AG inhibited 77.8 and 90.3% of DAO activity in cotyledon and embryo of germinating fava bean, respectively. Accordingly, 25.0 and 29.3% of GABA accumulation was inhibited. From Table 2, it can be found that in root or embryo of soybean or fava bean, DAO activity was inhibited up to 90% by AG; while in cotyledon, only 70-80%. In addition, the inhibitory action is inequable under different stresses. Fava bean seed, in contrast to soy bean, is larger and with thick seed coat, therefore, it is difficult for AG to penetrate into seed. This indicates that the effective inhibition of DAO activity in different plants is related to the size and composition of seeds. Based on this study, it can only be said that the polyamine degradation pathway provided, at least, 26.9 and 29.3% of GABA formation in, respectively, cotyledon and embryo of germinating fava bean under NaCl-hypoxia stress, as DAO activity was not inhibited completely.

Polyamines are the key substrates for GABA accumulation in polyamine degradation pathway. Under pathogen invasion (Yoda *et al.*, 2003) and abiotic stress, polyamine contents in plants increased significantly (Bouchereau *et al.*, 2009). In this study, the change trend of polyamine (especially Put and Spd) content is similar to that of Tian *et al.* (2005) and Liu *et al.* (2004) When treating with AG, especially Put content was accumulated significantly, because DAO mainly catalyzes Put although it could catalyze Spd slightly (Yang *et al.*, 2012). This indicates that Put is the main substrate of GABA under hypoxia-NaCl stress in germinating fava bean.

CONCLUSIONS

Under hypoxia-NaCl stress, polyamine degradation pathway provided approximately one third of GABA formation in germinating fava bean. Put is the main substrate of GABA in polyamine degradation pathway. In addition, the embolism of polyamine degradation pathway is all-important for plant growth.

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Table 2. Comparison of AG on DAO activity and GABA accumulation in different plants.

Plant	Growth condition	AG (mmol L^{-1})	Inhibitory of DAO activity (%)			Inhibitory of GABA formation (%)		
			Cotyledon	Root	Embryo	Cotyledon	Root	Embryo
Soybean (Xing *et al.*, 2007)	NaCl	1.0	—[b]	97.6	—	—	39	—
Soybean (Guo *et al.*, 2012)	Hypoxia	7.5	70.6	—	97.2	10.3	—	36.8
Fava bean (Yang *et al.*, 2011)	Non-stress	7.5	89.8[a]	—	—	31.2[a]	—	—
Fava bean[c]	Hypoxia-NaCl	7.5	77.7	—	90.3	25.0	—	29.3

[a] * Whole sprout , [b] Not determined, [c] Data in this research.

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مسیر تجزیه پلی آمین ها تنظیم کننده رشد و انباشت گاما آمینو بیوتیریک-
اسید(GABA) در بذر جوانه زده باقلا در شرایط کمبود اکسیژن و تنش کلرور سدیم

ر. یانگ، ی. یین و ز. گو

چکیده

در گیاهان، ساخت گاما آمینو بیوتیریک اسید(GABA) در مسیر تجزیه پلی آمین ها و نیز در مسیر GABA shunt انجام می شود. آمینو گوانیدین (AG) یک مهار کننده ویژه آنزیم کلیدی دی آمین اکسیداز DAO) برای تشکیل GABA در مسیر تجزیه پلی آمین ها می باشد. در پژوهش حاضر، AG به کار گرفته شد تا اثر مسیر تجزیه پلی آمین ها روی رشد و انباشت GABA در بذر جوانه زده باقلا در شرایط کمبود اکسیژن و تنش کلرور سدیم بررسی شود. نتایج نشان داد که ۵ میلی مول در لیتر AG فعالیت DAO را در حد اکثر (ولی نه به طور کامل) مهار کرد و نیز به طور همزمان رشد جوانه ها را متوقف کرد. به این قرار، جلوگیری از مسیر تجزیه پلی امین ها، به طور معنی داری رشد جوانه زنی باقلا را تحت تاثیر قرار داد. مسیر تجزیه پلی امین ها ۲۶/۹٪ و ۲۹/۳٪ GABA را به ترتیب در لپه و جنین فراهم کرد زیرا فعالیت DAO به طور کامل مهار نشده بود. پلی آمین ها، به ویژه پوتره سین (Put)، بعد از توقف مسیر تجزیه پلی آمین ها انباشته شد و این نشانه آن بود که Put بستره اصلی GABA در مسیر تجزیه پلی آمین ها است.