

Full Length Research Paper

# Effects of nitrogen forms on the growth, ascorbate-glutathione cycle and lipid peroxidation in developing seeds of vegetable soybean

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A pot culture experiment of nitrogen forms ( $\text{NO}_3^-$ : $\text{NH}_4^+$ ) with four ratios (100:0, 75:25, 50:50, and 25:75) in nutrient solutions was conducted to examine the effects of nitrogen forms on the growth, ascorbate-glutathione (ASC-GSH) cycle and lipid peroxidation in developing seeds of vegetable soybean. Results showed that the best plant growth vigor was observed in  $\text{NO}_3^-$  (75%), and then in  $\text{NH}_4^+$  (50%). The contents of ASC and dehydro-ascorbate (DHA), and DHA/ASC ratio in  $\text{NO}_3^-$  (75%) and  $\text{NH}_4^+$  (50%) maintained stable values, but obvious increases were observed in  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%). The GSH contents exhibited increasing trends, while the increases in oxidized glutathione (GSSG) contents were only observed in  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%), in which high GSSH/GSH ratios were observed. In  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%), the enzymes activities in ASC-GSH cycle, including ascorbate peroxidase (APX, EC 1.11.1.11), dehydro-ascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2), expressed increasing trends. Significant increases in lipid peroxidation and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) generation were also observed in  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%) as against  $\text{NO}_3^-$  (75%) and  $\text{NH}_4^+$  (50%). It was shown that the degree of oxidative damage was relatively low when the ratio of nitrogen forms was 75:25, and this could be a good guidance on the nitrogen application in the cultivation of vegetable soybean.

**Key words:** Nitrogen forms, seed development, ascorbate-glutathione cycle, vegetable soybean.

## INTRODUCTION

Nitrogen is a major element which is essential for plant growth, and soil nitrogen is available to plants in the form of either nitrate nitrogen ( $\text{NO}_3^-$ -N) or ammonium nitrogen ( $\text{NH}_4^+$ -N). Nitrogen forms affect plant growth and yield in both soil-based and soil-less grown crops (Forde and Clarkson, 1999). Generally, plant growth decreases under excessive nitrogen supplied, in which the growth of some plant species was inhibited significantly such as potato (Cao and Tibbitts, 1998), bean (Sánchez et al., 2004), and strawberry (Tabatabaie et al., 2006). Under high nitrogen levels, most plant species show reduced growth, smaller leaves and stunted root systems, and in severe cases can lead to death of the plant. High nitrate levels in soil or

nutrient solution will cause osmotic stress, which can cause oxidative damage and induce reactive oxygen species (ROS). ROS are highly toxic and can damage many important cellular components, such as lipids, protein, DNA and RNA (Wei et al., 2009). In addition, increased levels of ammonium were highly toxic for plant cells (Pilbeam and Kirkby, 1992). However, when exposed to high ammonium concentrations, plants will accumulate many low molecular mass osmolytes, such as sugars, proline, organic acids, polyamines etc. (Claussen et al., 2006) to become more tolerant.

It has been known that, in plant cells, the enhanced generation of ROS, potentially leading to oxidative stress, in response to both abiotic and biotic stresses had been well documented (Masood et al., 2006). Within the cellular mechanism protecting against the deleterious effects of ROS, ascorbate and glutathione seemed to play a fundamental role. The ASC and GSH, together with anti-

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oxidant enzymes: APX, DHAR and GR were involved in ROS scavenging (Asada, 1999; Kuniak and Skłodowska, 2001; Foyer and Noctor, 2003). APX, because of its presence in all cell compartments and its high affinity to  $H_2O_2$ , is an important enzyme participating in cell detoxification. The electron donor is ASC, and its acid pool is regenerated by DHAR, with the participation of the reduced form of GSH and nicotinamide adenine dinucleotide phosphate (NADPH). GSH is oxidized to the disulphide GSSG and may be reduced back to GSH by GR. ASC may directly react with the hydroxyl radical ( $OH^\cdot$ ), the superoxide anion radical ( $O_2^{\cdot-}$ ), and the singlet oxygen ( $^1O_2$ ), besides its participation in the ascorbate-glutathione pathway (Noctor and Foyer, 1998; Pukacka and Ratajczak, 2006). Moreover, ASC and GSH are also associated with the cellular redox balance and the ratios of DHA/ASC and GSSG/GSH may function as signals for the regulation of antioxidant mechanisms (Mittler, 2002).

Vegetable soybean (*Glycine max* [L.] Merr.) which contains about 16% protein, twice that of beans (Mookherji et al., 1991); and is also rich in minerals, vitamins A and B, is very popular in China, particularly in the southern region of the Yangtze River. In recent years, researches on vegetable soybean with respect to environmental stresses, such as drought, high salt and low temperature have been conducted but few reports have focused on the effects of nitrogen nutrition. According to Streeter (1981), high nitrogen was a key limiting factor in the processes of nitrogen fixation and nodule formation. Hungria and Vargas (2000) reported that the application of mineral nitrogen significantly reduced nodule number. Moreover, the existence of excessive ammonium in plant rhizosphere could further depress pH due to secretion of hydron originated from the nitrification process (Pilbeam and Kirkby, 1992; Savvas et al., 2003). The low pH decreased the nitrogen-fixing capabilities in soybean and resulted in significant reductions in yields (Franco and Munns, 1982).

Researches on the roles of nitrogen forms have been concentrated on the morphology and production of vegetable soybean, however, little is known about the physiological responses especially ascorbate-glutathione metabolism cycle and lipid peroxidation to different nitrogen forms in developing seeds of vegetable soybean. Therefore, the objectives of this experiment were to examine how antioxidants and enzymes react in ascorbate-glutathione metabolism cycle under different nitrogen forms, and to elucidate their possible roles in the defense reaction against excessive nitrate or ammonium in the seed development of vegetable soybean.

## MATERIALS AND METHODS

### Plant material and culture conditions

The experiment was conducted in the glasshouse of Nanjing Agricultural University, China. On the 8th of September, 2008, seeds of vegetable soybean [*G. max* (L.) Merr. cv. Li-xiang 95-1] were sterilized with sodium hypochlorite containing 5% active chloride for

5 min, soaked for 6 h in distilled water after being washed 5 times and then germinated at  $25 \pm 1^\circ C$  for 48 h on moist filter paper in Petri dishes (11 cm in diameter). The uniformly germinated seeds were selected, sowed in 48 plastic pots (60 × 45 × 45 cm [height, upper and lower diameters respectively]) filled with a 1:1 mixture of peat and vermiculite (5 seeds per pot). Plants were grown under natural light. Day/night temperatures were 28 - 30 / 20 - 22 °C. Relative humidity was between 60 and 80% while midday photosynthetic photon flux density was between 550 and 850  $mmol\ m^{-2}\ s^{-1}$  (September - November, 2008). Seedlings were thinned to 4 plants per pot after emergence. For treatments of nitrogen forms, the experiment was a completely randomized design with three replicates per treatment. Seven days later, each pot was irrigated every three days with 1 L of nutrient solution containing different nitrogen forms.

On October 5, flowers of vegetable soybean emerged, and they were labelled after anthesis. Twelve days after flowering (DAF), pods were collected at 3 days intervals until 30 DAF. At each stage, pods from different plants were picked and seeds from each pod were collected for antioxidant content and enzyme activity measurements.

### $NO_3^- : NH_4^+$ ratio treatment

The plants were fed with one of the following  $NO_3^- : NH_4^+$  ratios: 100:0, 75:25, 50:50, and 25:75. Modified Hoagland's solution (Hoagland and Arnon, 1950) was used to prepare the nutrient solution. The composition of the nutrients in the solutions was as follows (in mM): 7.7 K, 4.2 Ca, 2 Mg, 2 S, 1 P; (in mM): 140 B, 100 Cu, 36 Mn, 46 Zn, 30 Fe (Fe-EDTA), 1 Mo. Total nitrogen at 16 mM was

provided as  $NO_3^- N^-$  and  $NH_4^+ N^+$  to give  $NO_3^- : NH_4^+$  ratios of 100: 0, 75:25, 50:50, and 25:75 (Table 1). To obtain identical K, Ca, Mg, total N and P concentrations in all treatments, the changes in  $NO_3^- : NH_4^+$  ratios were balanced by varying the  $Cl^-$  concentration. The initial pH of the nutrient solutions containing nitrate and ammonium nitrogen was adjusted to 6.5 - 6.8 by adding 1M HCl or NaOH. The electrical conductivity (EC) of the modified Hoagland's solution was within the range of 2.6 - 2.8  $dS\ m^{-1}$ .

### Data collection and chemical analyses

#### Plant height, seed length and seed dry weight determination

As the plants grew, the growth parameters of vegetable soybean, including plant height (10, 20, and 30 days after sowing, DAS) and seed length (10, 20, and 30 days after flowering, DAF) were measured. At each sampling date, 5 plants were harvested from each replication; seeds were collected to weigh fresh weight. Subsequently, seeds were dried at 68°C for 96 h and re-weighed for dry weight.

#### Determination of ASC, DHA, GSH and GSSG concentrations

To determine the contents of ASC, DHA, GSH and GSSG, the frozen samples of seeds were homogenized in ice-cold 5% (w/v) TCA and then centrifuged at 12000 rpm for 20 min at 4°C. ASC and DHA contents were measured according to Arrigoni et al. (1992). This method was based on the reduction of  $Fe^{3+}$  to  $Fe^{2+}$  with ASC in acid solution followed by the formation of a red chelate between  $Fe^{2+}$  and bathophenanthroline, which absorbed at 534 nm. Total ascorbate was determined through a reduction of DHA to ASC by dithiothreitol (DTT) and then DHA content was calculated by subtracting the reduced ASC content from the total ascorbate. GSSG and total (GSH + GSSG) contents were estimated by the method of Anderson et al. (1992). The assay was based on sequential oxidation of GSH by

**Table 1.** The concentrations of inorganic salts (mM) used to prepare nutrient solutions at NO<sub>3</sub><sup>-</sup>-N: NH<sub>4</sub><sup>+</sup>-N ratios of 100:0, 75:25, 50:50 and 25:75.

Inorganic salts	Nitrogen treatment ratio of nitrate-N to ammonium-N in the nutrient solution			
	100 : 0	75 : 25	50 : 50	25 : 75
Ca(NO <sub>3</sub> ) <sub>2</sub>	4.3	2.7	3.6	1.8
KNO <sub>3</sub>	5.7	5.4	0.0	0.0
MgSO <sub>4</sub>	2.0	2.0	2.0	2.0
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	0.0	1.0	1.0	1.0
KH <sub>2</sub> PO <sub>4</sub>	1.0	0.0	0.0	0.0
KCl	1.0	2.3	7.7	7.7
NH <sub>4</sub> Cl	0.0	2.5	6.1	9.7
CaCl <sub>2</sub>	0.0	1.5	0.7	2.4

[5, 5'-dithiobis-(2-nitrobenzoic acid)] (DTNB) to produce TNB and reduction of GSSG by NADPH in the presence of GR. For specific assay of GSSG, the GSH could be masked by derivatisation with 2-vinylpyridine in the presence of triethanolamine for 1.5 h at 25°C according to the procedure of Pukacka and Ratajczak (2006). The change in absorbance at 412 nm was followed at 25°C, and a standard curve was prepared through the utilization of the GSH standard.

#### Determination of enzyme activities, APX, DHAR and GR

Seeds of vegetable soybean (200 mg) were homogenized in 1.6 ml and 50 mM sodium phosphate buffer (pH 7.8) containing 30 mM EDTA and 2% (w/v) PVP. The homogenate was centrifuged at 12000 rpm for 20 min at 4°C. The supernatant was used for APX activity as described by Nakano and Asada (1981). The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25

mM ascorbate, 1.0 mM H<sub>2</sub>O<sub>2</sub> and 100 l enzyme extract. The reaction was started by the addition of H<sub>2</sub>O<sub>2</sub> and ascorbate oxidation measured at 290 nm for 45 s. DHAR extraction was performed in a 50 mM sodium phosphate buffer (pH 7.2) containing 1 mM Na<sub>2</sub>EDTA, 0.05% cysteine (w/v) and 2% (w/v) PVP. DHAR activity was measured by following the formation of ASC from DHA at 265 nm according to Cakmak and Marschner (1992). GR activity was measured according to Foyer and Halliwell (1976). The extraction of GR was performed in a 50 mM Tricine-NaOH buffer (pH 7.8)

containing 1 mM Na<sub>2</sub>EDTA and 2% (w/v) PVP. The reaction mixture consisted of 950 l of NADPH, 0.05 mM GSSG, and 50 l extract. The reaction was started by the addition of GSSG and the NADPH oxidation rate was monitored at 340 nm for 3 min.

#### Determination of malondialdehyde (MDA) and H<sub>2</sub>O<sub>2</sub> levels

For the assay of MDA, 0.5 g fresh seeds was homogenized with 3 ml of 5% trichloroacetic acid (TCA) and centrifuged at 3000 rpm for 10 min at 4°C. The crude extract was mixed with the same volume of a 0.67% (w/v) thiobarbituric acid (TBA) solution containing 20% (w/v) TCA. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. After the tube was centrifuged at 3000 rpm for 10 min, the absorbance of the supernatant was monitored at 532 and 600 nm. After subtracting the non-specific absorbance (600 nm), the MDA concentration was determined by its molar extinction coefficient (155 mM<sup>-1</sup>·cm<sup>-1</sup>) and the results were expressed as mol MDA g<sup>-1</sup> FW. For the assay of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 0.5 g fresh seeds was homogenized with 5 ml of 5% TCA containing 10 mmol·L<sup>-1</sup> ethylenediaminetetraacetic (EDTA). The homogenate was filtered

through two layers of cheesecloth and then centrifuged at 12,000 rpm for 15 min at 4°C. The total amount of supernatant was analyzed using the ferrithiocyanate method according to Sagisaka (1976). All spectrophotometric analyses were conducted on a spectrophotometer (Shimadzu UV-1601, Japan).

#### Statistical analyses

Significances were tested by one-way ANOVA followed by Duncan's test at P < 0.05 by SPSS 12 software (SPSS Inc., Chicago, U. S. A.), and the results were expressed as the mean values. The graphs were drawn by using Microsoft Excel software.

## RESULTS

### Effects of nitrogen forms on plant growth

The plant height, seed length, seed dry weight and percentage of seed dry weight of vegetable soybean as a function of various nitrogen forms in the nutrient solution are given in Tables 2 and 3. At the early stage (10 and 20 days after sowing, DAS), nitrogen forms had no significant effects on plant height, except in NH<sub>4</sub><sup>+</sup> (75%) at 20 DAS, in which the plant height significantly decreased compared with other treatments. Among the three stages, the highest plant height was observed at 30 DAS, and the plant height in NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%) was significantly higher than that in other treatments, in which the lowest value was found in NH<sub>4</sub><sup>+</sup> (75%), 60.7 cm. In seed length, during the first 10 days after flowering (DAF), the value in either NO<sub>3</sub><sup>-</sup> (75%) or NH<sub>4</sub><sup>+</sup> (50%) was significantly higher than that in NH<sub>4</sub><sup>+</sup> (75%), but no significant differences were found between NO<sub>3</sub><sup>-</sup> (100%) and other treatments. At 20 DAF, the highest seed length was observed in NO<sub>3</sub><sup>-</sup> (75%), 12.1 mm, and was significantly higher than that of other treatments; however, the lowest value was found in NH<sub>4</sub><sup>+</sup> (75%), 7.5 mm. No significant differences were observed between NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (50%) treatments. Among the sampling dates, like plant height, the highest seed length was observed at 30 DAF, and the values of seed length in NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%) were significantly higher than those in other treatments, and the

**Table 2.** Effects of nitrogen forms on the plant height and seed length at different stages of vegetable soybean.

Treatment (NO <sub>3</sub> <sup>-</sup> :NH <sub>4</sub> <sup>+</sup> )	Plant height (cm)			Seed length (mm)		
	10 DAS	20 DAS	30 DAS	10 DAF	20 DAF	30 DAF
100 : 0	13.1 a	43.6 a	67.1 b	4.1 ab	9.9 b	11.1 b
75 : 25	13.4 a	44.9 a	72.5 a	4.6 a	12.1 a	14.2 a
50 : 50	13.9 a	44.2 a	71.4 a	4.4 a	10.8 b	13.0 a
25 : 75	13.3 a	39.5 b	60.7 c	3.5 b	7.5 c	8.9 c

Note: Different letters within the same column indicate significant difference at 5% level. DAS: days after sowing; DAF: days after flowering.

**Table 3:** Effects of nitrogen forms on the seed dry weight and its dry weight percentage at different stages of vegetable soybean.

Treatment (NO <sub>3</sub> <sup>-</sup> :NH <sub>4</sub> <sup>+</sup> )	Seed dry weight (g/100 seeds <sup>-1</sup> )			Percentage of seed dry weight (%)		
	10 DAF	20 DAF	30 DAF	10 DAF	20 DAF	30 DAF
100 : 0	1.97 a	6.81 b	16.76 c	29.37 a	23.19 c	26.93 b
75 : 25	2.01 a	7.43 a	22.51 a	29.44 a	27.76 a	32.12 a
50 : 50	2.05 a	7.29 a	19.38 b	29.36 a	25.62 b	29.57 a
25 : 75	1.99 a	6.17 c	13.94 d	29.41 a	22.98 c	25.16 b

Note: Different letters within the same column indicate significant difference at 5 % level. DAF: days after flowering.

lowest value was found in NH<sub>4</sub><sup>+</sup> (75%), 8.9 mm.

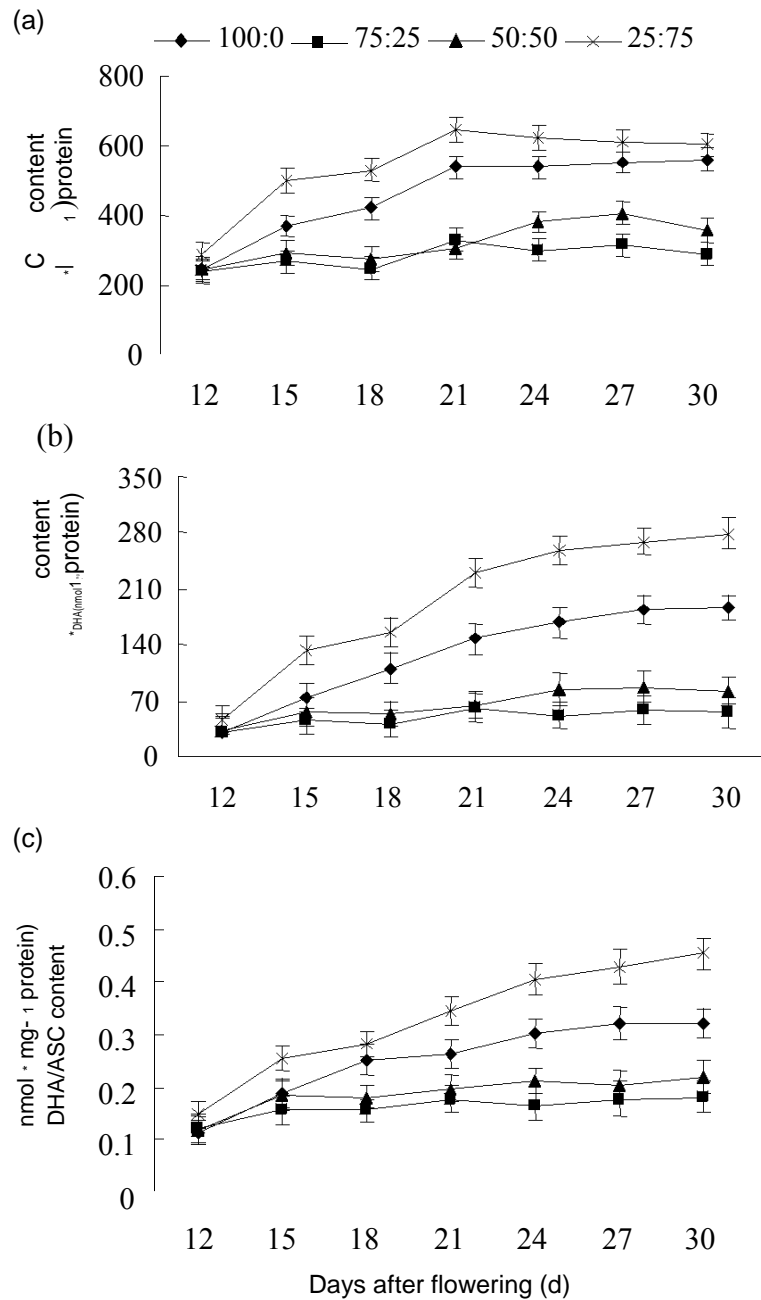
Seed dry weight was significantly affected by nitrogen forms at 20 and 30 days after flowering. At 20 DAF, no significant differences in seed weight were found between NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%), but seed dry weight was decreased significantly in NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%), in which the lowest value was observed in NH<sub>4</sub><sup>+</sup> (75%), 6.17 g×100 seeds<sup>-1</sup>. Under the treatments of different nitrogen forms, the differences in seed dry weight were significant at 30 DAF. The highest and lowest values were observed in NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> (75% each), 22.51 and 13.94 g×100 seeds<sup>-1</sup>, respectively. Nitrogen forms had no significant effects on percentage of seed dry weight at 10 DAF, however, the effects became obvious at 20 and 30 DAF. At 20 DAF, the percentage of seed dry weight in NO<sub>3</sub><sup>-</sup> (75%) was significantly higher than that in other treatments, and the values in NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%) were significantly lower than those in NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%). At 30 DAF, no significant differences in percentage of seed dry weight were found between NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%), in both of which the percentages of seed dry weight were significantly higher than those in NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%).

### Effects of nitrogen forms on the ascorbate and glutathione contents

The influences of nitrogen forms on ASC and DHA contents, and DHA/ASC ratio in vegetable soybean seeds are shown in Figure 1 (a-c). Under NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%) treatments, ASC contents were maintained at 300

nmol×mg<sup>-1</sup> protein throughout the sampling dates (12 to 30 DAF). However, under NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%) treatments, there were steep increases by 292.4 and 359.6 nmol×mg<sup>-1</sup> protein, respectively, in the ASC contents between 12 and 21 DAF; thereafter the ASC contents were maintained at high levels till 30 DAF. DHA calculated in terms of the difference between total and reduced ascorbate contents in NH<sub>4</sub><sup>+</sup> (75%) was more than 4-fold the amount found in NO<sub>3</sub><sup>-</sup> (75%) from 21 to 30 DAF. DHA/ASC ratio increased in NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%) during the sampling dates (12 to 30 DAF). DHA/ASC ratios under NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%) treatments were 0.13 and 0.16 at 12 DAF and they reached their maximum 0.33 and 0.46 at 30 DAF, respectively and were significantly higher than those in NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%) treatments from 18 to 30 DAF.

As shown in Figure 2 (a-c), under different nitrogen forms, the changes in GSH content exhibited increasing trends, but the increases in NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%) treatments were obvious. GSH levels were highest at 27 DAF under both nitrogen forms treatments. In the four treatments, the highest value was observed in NH<sub>4</sub><sup>+</sup> (75%); 55.5 nmol×mg<sup>-1</sup> protein, followed by NO<sub>3</sub><sup>-</sup> (100%); 46.9 nmol×mg<sup>-1</sup> protein, and the lowest value was observed in NO<sub>3</sub><sup>-</sup> (75%); 28.4 nmol×mg<sup>-1</sup> protein. Levels of GSSG were almost 10 to 20-fold lesser in comparison to GSH contents under all treatments. Unlike the treatments of NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%), in which the GSSG levels were maintained at a stable value of 3 nmol×mg<sup>-1</sup> protein, the GSSG levels increased steadily during the whole sampling course (12 to 30 DAF) in NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%). At 30 DAF, the GSSG levels in NO<sub>3</sub><sup>-</sup> (75%) and



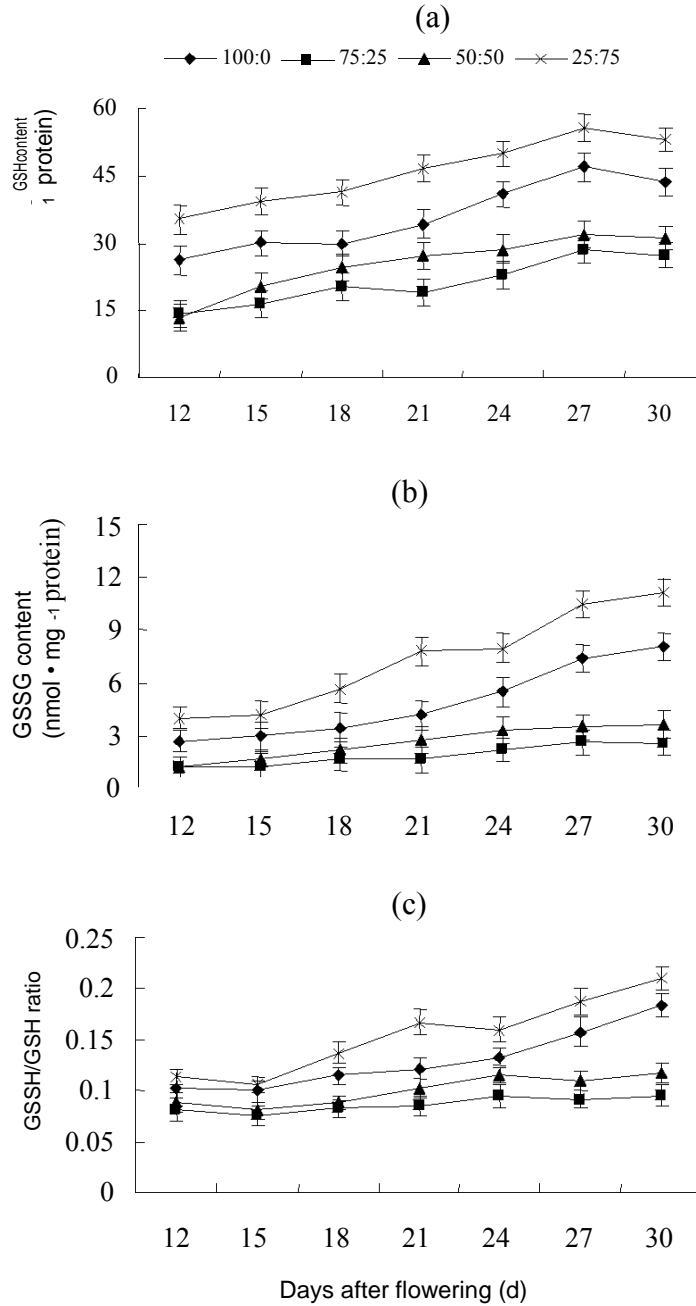
**Figure 1.** (a) Changes in levels of ascorbate; (b) dehydro-ascorbate and; (c) ratio of DHA/ASC in developing seeds of vegetable soybean under different nitrogen forms. Significances were tested by one-way ANOVA, and the results were expressed as the mean values  $\pm$  S.D; n=3.

$\text{NH}_4^+$  (50%) were almost 2- and 3-fold lower compared to those in  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%), respectively. GSSH/GSH ratios were 0.10 and 0.11 at 12 DAF and they increased to 0.18 and 0.21 at 30 DAF under  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%) treatments, respectively. The increasing rate of the GSSH/GSH ratio was much faster in  $\text{NH}_4^+$  (75%) than that in  $\text{NO}_3^-$  (100%). However, the rate of increase in the GSSH/GSH ratio was lesser in  $\text{NO}_3^-$  (75%) and  $\text{NH}_4^+$

(50%) compared with  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%) treatments.

#### Effects of nitrogen forms on enzymatic activities in ASC-GSH cycle

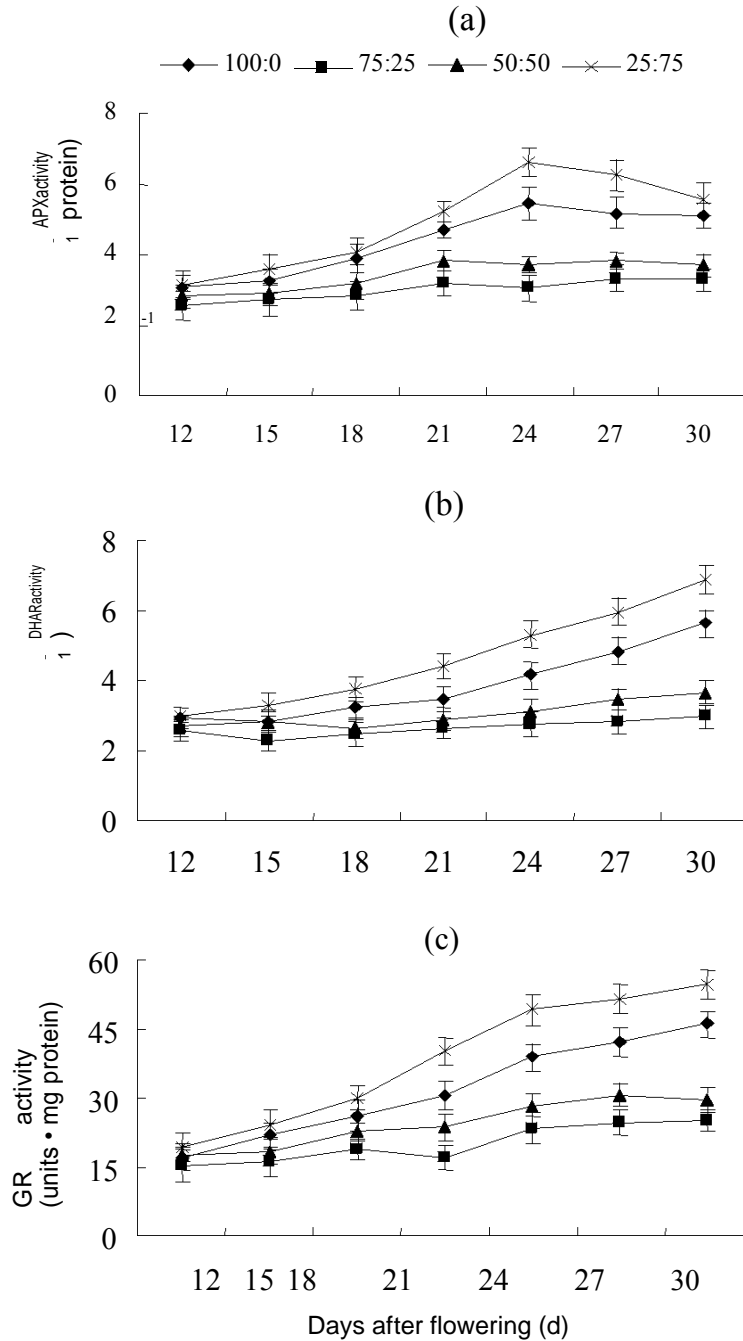
The influence of nitrogen forms on the activities of APX, DHAR and GR in seeds of vegetable soybean is shown in



**Figure 2.** (a) Changes in levels of reduced glutathione (GSH); (b) oxidized glutathione (GSSH) and; (c) ratio of GSSH/GSH in developing seeds of vegetable soybean under different nitrogen forms. Significances were tested by one-way ANOVA, and the results were expressed as the mean values  $\pm$  S.D; n=3.

Figure 3 (a-c). Under  $\text{NO}_3^-$  (75%) and  $\text{NH}_4^+$  (50%) treatments, the activities of APX displayed slight increases in the initial 9 days (12 to 21 DAF) and then, maintained at stable values in the next 9 days (21 to 30 DAF). However, under  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%) treatments, the increases in APX activity were more obvious, especially

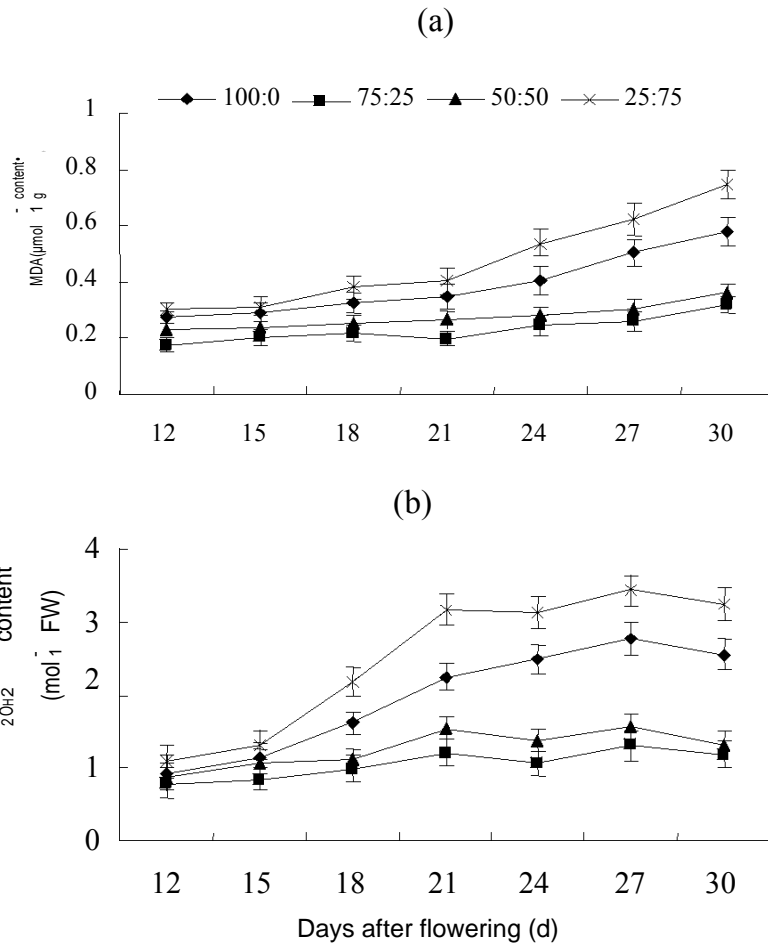
from 21 to 30 DAF, and the highest values were observed at 24 DAF (5.4 units $\times$ mg<sup>-1</sup> protein in  $\text{NO}_3^-$  (100%) and 6.6 units $\times$ mg<sup>-1</sup> protein in  $\text{NH}_4^+$  (75%), respectively). Among the sampling dates (12 to 30 DAF), increasing trends in DHAR activities were found, but the increases in  $\text{NH}_4^+$  (75%) and  $\text{NO}_3^-$  (100%) were more rapid and significantly



**Figure 3.** (a) Changes in activities of ascorbate peroxidase (APX); (b) dehydro-ascorbate reductase (DHAR) and; (c) glutathione reductase (GR) in developing seeds of vegetable soybean under different nitrogen forms. Significances were tested by one-way ANOVA, and the results were expressed as the mean values  $\pm$  S.D; (n=3).

and significantly higher than those in  $\text{NO}_3^-$  (75%) and  $\text{NH}_4^+$  (50%), especially from 21 and 30 DAF. The highest activities were observed at 30 DAF, and in  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%) the activities were approximately 1.9 and 2.3-fold higher than those in  $\text{NO}_3^-$  (75%), respectively. Like DHAR, GR activities were also increased in all treatments

from 12 to 30 DAF. However, under  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%) treatments, the increases in GR activity were significantly higher in the initial 12 days (12 to 24 DAF) in comparison with other treatments and then the increase slowed down in the next 6 days (24 to 30 DAF). At 30 DAF, the highest GR activity in  $\text{NH}_4^+$  (75%) was found to be



**Figure 4.** (a) Changes in levels of malondialdehyde (MDA); (b) hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in developing seeds of vegetable soybean under different nitrogen forms. Significances were tested by one-way ANOVA, and the results were expressed as the mean values  $\pm$  S.D; (n=3).

1.8- and 2.2-fold higher than that in NH<sub>4</sub><sup>+</sup> (50%) and NO<sub>3</sub><sup>-</sup> (75%), respectively.

#### Effects of nitrogen forms on MDA and H<sub>2</sub>O<sub>2</sub> levels

The effects of nitrogen forms on levels of lipid peroxidation (expressed as changes in content of MDA) in vegetable soybean seeds are shown in Figure 4a. Among the sampling dates, there were slightly increasing trends of MDA levels in seeds grown in NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%), and no significant differences were found between them. However, the increasing trends were obvious in NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%), and the MDA levels were significantly higher than those in NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%) especially from 21 to 30 DAF. As shown in Figure 4b, among the sampling dates, no obvious changes were found in H<sub>2</sub>O<sub>2</sub> levels between NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%), in which the levels were 1-1.5 mol·g<sup>-1</sup> FW. However, in the initial 9 days after flowering, the H<sub>2</sub>O<sub>2</sub> levels in NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%) increased significantly, and was then maintained at high levels

from 21 to 30 DAF. At 30 DAF, the H<sub>2</sub>O<sub>2</sub> level in NH<sub>4</sub><sup>+</sup> (75%), was found to be approximately 3-fold than that in NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%).

#### DISCUSSION

The effectiveness of nitrogen forms in the present experiment was reflected in the growth parameters (Tables 2 and 3). Under high nitrate (100%) and ammonium (75%), the growth and biomass production of plants and seeds were strongly depressed compared with other treatments, especially 30 days after sowing (DAS) in plant height and 20 to 30 days after flowering (DAF) in seeds. This phenomenon might be attributed to the acid-base balance that was broken, in which NO<sub>3</sub><sup>-</sup> increased the pH around the roots due to the efflux of HCO<sub>3</sub><sup>-</sup> or OH<sup>-</sup>, and NH<sub>4</sub><sup>+</sup> decreased the pH due to the efflux of H<sup>+</sup> (Bar and Kafkafi, 1992; Romero et al., 2006).



He et al. (2007) reported that high  $\text{NO}_3^-$  concentration resulted in nitrite production which was converted into nitric oxide (NO) in plants, while NO and  $\text{O}_2^-$  could be rapidly catalyzed by nitrate reductase (NR) into peroxynitrite ( $\text{ONOO}^-$ ), which is highly toxic to plants and inhibits plant growth. Also, the less growth of plants fed with high concentration of  $\text{NH}_4^+$  might be due to the unavailability of  $\text{NO}_3^-$  as a nitrogen source and the higher demand of carbohydrates channelled for  $\text{NH}_4^+$  assimilation and detoxification (Nathawat et al., 2007; Tabatabaei et al., 2008). Results of the current work showed good morphological development as well as seeds production in  $\text{NO}_3^-$  (75%) and  $\text{NH}_4^+$  (50%).

Under 25:75 ( $\text{NO}_3^-$  :  $\text{NH}_4^+$ ) treatment, the plant height, seed length and seed dry weight were significantly lower than those of other treatments. Walch-Liu et al. (2000) reported that excessive  $\text{NH}_4^+$  was harmful to plants and could result in hormonal imbalance and a strong decline of cytokinins in the xylem sap, which could then hamper growth and reduce yield. In addition, Gerendas et al. (1997) believed that the high concentration of  $\text{NH}_4^+$  in nutrient solution could lead to  $\text{NH}_4^+$  toxicity, which was considered to result in effects such as  $\text{NH}_4^+$  induced nutrient deficiency caused by the impaired uptake of ions, acidification of metabolism, damage of antioxidant enzyme system and cell membrane integrity. Ammonium toxicity was related with potential difference across the plasma membrane, and the capacity of ammonium assimilation (Magalhaes and Huber, 1991).

Generally, it has been described that nitrogen toxicity in plants was accompanied by a reduction in growth. This effect was explained by the elevated energy consumption caused by the transport costs of excessive nitrate or ammonium (Britto et al., 2001) and the decreased protein and sugar contents as described by Cao et al. (2004). The results of the present study suggested an additional and not alternative explanation to growth inhibition caused by the broken ROS imbalance in seeds exposed to excessive nitrate or ammonium; however further studies coupling molecular level measurements would be necessary.

Under favorable growth conditions, the reactive oxygen species (ROS) production and elimination maintained a relative balance in plants (Liu et al., 2007). In this study, fluctuations around a relative stable value in the antioxidative enzymes and metabolites observed in  $\text{NO}_3^-$  (75%) and  $\text{NH}_4^+$  (50%) could have resulted from the fine metabolic regulation performed by the ROS cycle in terms of increasing active scavenging or suppressing metabolic activity responsible for ROS production. More specifically, ASC stimulates cell cycle activity and DHA blocks the normal cell cycle progression. In this context the time course changes in these compounds under  $\text{NO}_3^-$  (75%) and  $\text{NH}_4^+$  (50%) treatments; this could have been due to a cellular machinery regulating cell cycle.

Oxidative damage in plant tissues was alleviated by a concerted action of both enzymatic and non-enzymatic

antioxidant metabolisms (Hasegawa et al., 2000). Thus, efficient destruction of  $\text{H}_2\text{O}_2$  in plant cells required the concerted action of antioxidants. Among the non-enzymatic antioxidants, ASC was found to be one of the best characterized compounds required for many key metabolic functions in plant cells. In the initial few days after flowering (12 to 21 DAF), ASC contents in both  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%) treatments were enhanced with more significant increases in DHA levels, and then DHA/ASC ratios were clearly increased (Figure 1; a-c), suggesting that excessive nitrate or ammonium would stimulate the synthesis of ASC pool in developmental seeds of vegetable soybean, which attests to a defense reaction of the cells. Furthermore, ASC content and DHA/ASC ratio both in  $\text{NO}_3^-$  (100%) and in  $\text{NH}_4^+$  (75%) were significantly higher than those in  $\text{NO}_3^-$  (75%) and  $\text{NH}_4^+$  (50%), with significantly higher DHA levels especially from 18 to 30 DAF. The higher levels of ASC in  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%) might be ascribed to a corresponding increase in DHAR activity (Figure 3b), indicating that the defense mechanism of ASC in ascorbate-glutathione system was strengthened by excessive nitrate or ammonium. It has been reported that, the accumulation of DHA could bring about deleterious effects because it inhibits the activities of several enzymes of the chloroplastic metabolism in vitro (Ku niak and Skłodowska, 2001), and the increase in ASC content would play an important role in preserving APX activity (Chaparzadeh et al., 2004). Also, significantly higher ASC contents in  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%) might also have resulted from a concurrent enhancement in the GSH pool (Figures 1 and 2). Noctor and Foyer (1998) reported that both ASC and GSH, the low molecular weight antioxidants, could directly scavenge ROS or could cooperate with enzymatic scavengers. ASC and GSH are the major redox buffers in plant cells, where a decrease in their redox statuses leads to a loss of cell redox homeostasis (Foyer and Noctor, 2003). Under  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%) treatments, the contents of GSH and GSSG showed an increase trend in the initial few days after flowering, with no difference between  $\text{NO}_3^-$  (75%) and  $\text{NH}_4^+$  (50%) (Figure 2), suggesting a similar stimulating effect on synthesis of GSH as the trend of ASC level in seeds of vegetable soybean under excessive nitrate or ammonium. GSH and GSSG/ GSH ratio in  $\text{NO}_3^-$  (100%) and  $\text{NH}_4^+$  (75%) were significantly higher than those in  $\text{NO}_3^-$  (75%) and  $\text{NH}_4^+$  (50%), indicating that seeds under excessive nitrate or ammonium had a higher level of GSH pool, which might partly be affected by the enhancement of GR activity (Figure 3c). As shown by Liu et al. (2007), enhanced levels of ASC and GSH in ramie adapted to Cd stress indicated their active participation in detoxification of ROS directly, as well as through the ascorbate-glutathione cycle. By contrast, transgenic plants with changed levels of glutathione (Creissen et al, 1999) and mutants with inhibited ascorbate contents were found to be susceptible to stress conditions (Liu et al., 2007). In this sense, the

effect of GSH pool level on H<sub>2</sub>O<sub>2</sub> scavenging in ascorbate-glutathione metabolism system was more efficient under excessive nitrate or ammonium.

Several studies have shown that excessive nitrate or ammonium can induce the activity of antioxidative enzymes such as SOD, POD, CAT, APX and GR in plants (Zhu et al., 2000; Medici et al., 2004; Cao et al., 2004; Nimptsch and Pflugmacher, 2007), suggesting that excessive nitrate or ammonium can result in oxidative stress in plants. The results of this study supports that the production of excessive nitrate or ammonium-induced ROS might trigger the response of antioxidative defensive systems against oxidative stress, and contribute to metabolism disorder, including energy-consumption, decreased protein and sugar contents, and thus cause the reduction of yields or even death of plants.

As "ROS balancing" is a continuous process, lipid peroxidation products are always present. This was partly demonstrated by the results in this study obtained for APX, DHAR and GR activities in NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%) treatments (Figure 3). Under excessive nitrate or ammonium, NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%), the activities of APX, DHAR and GR were increased obviously, and these increases could be attributed to a higher capacity of the plant to detoxify hydroperoxides generated via lipid peroxidation caused by a surplus of ROS. This increased biotransformation capacity was reflected in the results of lipid peroxidation, where low levels of MDA were detected in vegetable soybean seeds exposed to excessive nitrate or ammonium, especially in the early days after flowering (12 to 21 DAF). Similar to the present study, elevated activities had been reported in common bean under ammonium stress (Zhu et al., 2000). The changes in contents of ASC and GSH showed a similar pattern in response to excessive nitrate or ammonium (Figures 1a and 2a), and the increased ASC content might be caused by elevated GR activity. These results suggested that the activities of enzymes and contents of metabolic antioxidants in the ascorbate-glutathione pathway were regulated actively in seeds of vegetable soybean in response to the induced-oxidative stress by excessive nitrate or ammonium.

The levels of MDA and H<sub>2</sub>O<sub>2</sub> were higher in seeds of vegetable soybean (Figure 4; a-b) under excessive nitrate or ammonium than those in NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%) however, the increases in H<sub>2</sub>O<sub>2</sub> were more significant, especially from 18 to 30 DAF. A possible explanation is that the increased antioxidative response alleviated or prevented lipid peroxidation, but the antioxidative response could not prevent the damage to vegetable soybean seeds when exposed to excessive nitrate, ammonium or long-term exposure. When plants were exposed to environmental stresses such as salinity, drought and high temperature, this balance would be disrupted, producing excessive ROS (Jin et al., 2003). In the absence of any protective mechanism, ROS could seriously disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids and eventual-

ly lead to cell death.

In conclusion, the present study has shown that the appropriate ratio of nitrogen forms could increase biomass production significantly, especially in NO<sub>3</sub><sup>-</sup> (75%). Under excessive nitrate and ammonium, NO<sub>3</sub><sup>-</sup> (100%) and NH<sub>4</sub><sup>+</sup> (75%), the developing seeds of vegetable soybean had relatively higher ascorbate and glutathione pool and activities of enzymes in ascorbate-glutathione cycle than those in NO<sub>3</sub><sup>-</sup> (75%) and NH<sub>4</sub><sup>+</sup> (50%) however, the high antioxidant capacities could not avoid the damage of excessive nitrate or ammonium, as shown by the growth characters of plants. The data presented in this experiment suggests that nitrogen forms significantly affected the growth and yield of vegetable soybean, and the ascorbate-glutathione cycle might play a fundamental role in the protective mechanism of developmental vegetable soybean seeds under excessive nitrate or ammonium. The appropriate ratio of 75:25 (NO<sub>3</sub><sup>-</sup> : NH<sub>4</sub><sup>+</sup>) could be a good guidance on nitrogen application in the cultivation of vegetable soybean.

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