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Changes in photosynthesis, fluorescence, and nitrogen metabolism of hawthorn (*Crataegus pinnatifida*) in response to exogenous glutamic acid

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Abstract

Photosynthesis, chlorophyll (Chl) *a* fluorescence, and nitrogen metabolism of hawthorn (*Crataegus pinnatifida* Bge.), subjected to exogenous L-glutamic acid (GLA) (200 mg l^{-1}, 400 mg l^{-1}, and 800 mg l^{-1}) that possibly affect secondary metabolic regulation, were measured. The results indicated that photosynthetic and fluorescence characteristics of hawthorn exhibited positive responses to the application of GLA. Different concentrations of GLA caused an increase in Chl content, net photosynthetic rate (P_N) and stomatal conductance (g_s) as well as transpiration rate (E), and improved the carboxylation efficiency (CE), apparent quantum yield (AQY) and maximum carboxylation velocity of Rubisco (V_{cmax}). Application of GLA could also enhance the maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PSII (F_v/F_0), the maximal quantum yield of PSII (F_v/F_m), the probability that an absorbed photon will move an electron into the electron transport chain beyond Q_A (Φ_{Eo}) as well as the performance index on absorption basis (PI_{ABS}), but decreased the intercellular CO_2 concentration (C_i) and the minimal fluorescence (F_0). Application of GLA also induced an increase in nitrate reductase (NR; EC 1.6.6.1) and glutamine synthetase (GS; EC 6.3.1.2) activities, and increased the soluble protein content, leaf nitrogen (N) content and N accumulation in leaves as well as the plant biomass. However, the effects were different among different concentrations of GLA, and 800 mg l^{-1} GLA was better. This finding suggested that application of GLA is recommended to improve the photosynthetic capacity by increasing the light energy conversion and CO_2 transfer as well as the photochemical efficiency of PSII, and enhanced the nitrogen metabolism and growth and development of plants.

Additional key words: chlorophyll fluorescence; glutamine synthetase; leaf nitrogen content; net photosynthetic rate; nitrate reductase; soluble protein content; stomatal conductance; transpiration rate.

Introduction

Amino acids are the precursor substances that are modified by a wide range of different enzymes to produce secondary metabolites such as flavonoids, alkaloids, and other nitrogen compounds. The chemical structure and composition of secondary metabolites are closely related to environmental conditions (Yan *et al.* 2007). Each amino acid can be used as an amino donor, but the most important amino acid is glutamic acid, the formation and transformation of which plays a very important role in the synthesis of amino acids and nitrogen compounds.

It is reported that plants can not only absorb inorganic nitrogen, but also can absorb and utilize the amino acids directly (Yin 1999, Liu *et al.* 2004). The various roles of amino acids in relation to crop growth have been studied

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Abbreviations: ABS – absorption flux; AQY – apparent quantum yield; C_a – atmosphere CO_2 concentration; CE – carboxylation efficiency; C_i – intercellular CO_2 concentration; Chl – chlorophyll; E – transpiration rate; ET_0/RC – electron transport efficiency per reaction center; F_0 – minimal fluorescence of dark adapted state; F_m – maximal fluorescence at dark-adapted state; F_v – variable fluorescence; F_v/F_0 – maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PSII; F_v/F_m – maximal quantum yield of PSII; g_s – stomatal conductance; GLA – L-glutamic acid; GLA_{200} – GLA concentration of 200 mg l^{-1}, GLA_{400} – GLA concentration of 400 mg l^{-1}, and GLA_{800} – GLA concentration of 800 mg l^{-1}; GS – glutamine synthetase; L_s – stomatal limitation value; NR – nitrate reductase; PI_{ABS} – performance index on absorption basis; P_N – net photosynthetic rate; PSII – photosystem II; Q_A – primary quinone electron acceptor of PSII; RC – reaction centers; V_{cmax} – maximum carboxylation velocity of Rubisco; Φ_{Eo} – the probability that an absorbed photon will move an electron into the electron transport chain beyond Q_A.

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extensively (Kirakosyan *et al.* 2004, Yang *et al.* 2005). Yin (1999) has found that appropriately higher amino acids content in tobacco can improve quality and flavour. Wang *et al.* (2006) have pointed out that application of exogenous GLA can promote anthocyanin accumulation in 'Fuji' apple. These are examples of amino acids being utilized to improve secondary metabolic pathways and synthesis efficiency.

Hawthorn (*Crataegus pinnatifida* Bge.) is a good source of flavonoids and its leaves contain rutin, hyperin, pigments, and other secondary metabolites. These secondary metabolites synthesized in hawthorn have recently received more attention, especially due to their vasoactive properties and antioxidant activity (Rakotarison *et al.* 1997, Kirakosyan *et al.* 2003). In recent years, the flavonoid ingredients contained in hawthorn leaves have been used in a wide range of developments and applications. However, basic research on flavonoids with regard to secondary metabolite regulation is comparatively less advanced. Despite the relatively great number of reports on the medicinal aspects and growth regulator effects on hawthorn (Rakotarison *et al.* 1997, Kirakosyan *et al.* 2003), there are only a few ones that attempt to explain the physiological basis of secondary metabolite regulation (Yan *et al.* 2007, Wang *et al.* 2006).

Photosynthetic capacity is positively correlated with leaf N concentration (Niu *et al.* 2003, Xu and Zhou 2005, Chen *et al.* 2005). However, the effects of exogenous GLA on photosynthesis, fluorescence and the key enzymes related to nitrogen metabolism have received little attention to date. Nitrate reductase (NR) and glutamine synthetase (GS) are key enzymes associated with nitrogen metabolism (Lam *et al.* 1996); they are also involved in photosynthesis and carbohydrate metabolism (Solomonson and Barber 1990, Sibout and Guerrier 1998). Unfortunately, the effects of GLA on the key enzyme activities related to nitrogen metabolism are still not well understood. To the best of our knowledge, no information on the photosynthetic physiological response in terms of photosynthetic physiology metabolism of hawthorn to exogenous GLA is available. The objective of this study was to investigate the effect of different concentrations of GLA on hawthorn leaf gas exchanges, Chl fluorescence characteristics and nitrogen metabolism, and to provide a theoretical basis for secondary metabolic regulatory mechanisms of hawthorn.

Materials and methods

The natural profile of study site: The study was conducted at Shenyang Agricultural University in China (42°N, 123°E, 50 m a.s.l.), with yearly sunshine hours around 2,587.6, and average temperature between 7 and 7.9°C. The maximum and minimum temperatures are 35.3 and –32.3°C, respectively. The annual frost-free period is about 153 days. The average annual precipitation is 705.4 mm with a temperate continental monsoon climate.

Experimental materials were obtained from one-year-old root suckers of hawthorn (C. *pinnatifida* Bge.), planted in nutrition pots in early May, 2007. The upper bore and pot height were 12.5 and 13 cm, respectively. The cultivation substrate was the mixture of garden soil, slag, and well-rotted feces (2:1:1, v:v:v). Forty randomly selected plants with fully expanded leaves and conventional fertilizer and water management were subjected to GLA treatments. 10 ml of GLA solutions of concentrations of 200, 400, and 800 mg l^{-1} (GLA_{200}, GLA_{400}, and GLA_{800}, respectively), and distilled water as a control were sprayed on the leaves without surfactant every 10 d in the morning since August 19, 2007.

Measurement of leaf gas exchange: Gas exchange was measured on seven fully expanded exposed leaves at the top of shoot (one leaf each plant). P_N, g_s, C_i, and E were simultaneously recorded every 7 d from 0 d to 28 d after application of GLA on August 19, 2007 with a portable photosynthetic system (*CIRAS-1*, *PP Systems*, Hitchin, UK) under uniform conditions (25°C, 350–360 μmol(CO_2) mol^{-1}, 75% RH). The irradiance of sample when P_N was measured was 1,413 μmol m^{-2} s^{-1} PAR. The stomatal limitation value (L_s) was calculated using the following formula: $L_s = 1 - C_i/C_a$ according to Yin *et al.* (2006). Linear regressions of irradiance and P_N over the range of 0–200 μmol(photon) m^{-2} s^{-1} of PAR were applied to determine the apparent quantum yield of carbon assimilation (AQY) (Yin *et al.* 2006). The slope of P_N-C_i response curve was the carboxylation efficiency (CE) = dP_N/dC_i. Maximum carboxylation velocity of Rubisco (V_{cmax}) was made by fitting a maximum likelihood regression below and above the inflexion of the assimilation *vs.* intercellular CO_2 concentration response using the method of McMurtrie and Wang (1993).

Chl *a* fluorescence parameters were measured on the same leaves as gas exchange using a Plant Efficiency Analyzer (*PEA*, *Hansatech*, King's Lynn, UK) after 20-min dark adaptation. The minimal fluorescence of dark-adapted state (F_0), the maximal fluorescence (F_m), the maximal quantum yield of PSII (F_v/F_m), maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PSII (F_v/F_0), the probability that an absorbed photon will move an electron into the electron transport chain beyond Q_A (Φ_{Eo}), and performance index on absorption basis (PI_{ABS}) were obtained or calculated from the instrument directly (Strasser and Strasser 1995). The variation of fluorescence was measured 7 times from 0 d to 28 d after application of GLA.

Soluble protein content and the activities of key enzymes: The youngest and fully expanded leaves were sampled every 7 d after application of GLA. The leaf fresh samples were obtained at about 9:00 h, and instantly frozen in liquid N for 1 min, then stored at –80°C for measurement of soluble protein and the key enzyme activity. About 1 g of leaves was homogenized with 10 ml of 50 mM sodium phosphate, pH 7.8, containing 2 mM EDTA and 80 mM L-ascorbic acid. After the homogenate was centrifuged at 15,000 × *g* for 20 min, the supernatants were used to determine soluble protein, and the activities of the key enzymes (Cruz *et al.* 1970). Soluble protein contents in leaves were determined according to the method of Bradford (1976). The activity of nitrate reductase (NR, EC 1.6.6.1) was determined according to the procedure of Abd-El Baki *et al.* (2000). The reaction medium consisted (total volume 1 ml) of 50 mM sodium phosphate, pH 7.8, 10 µM FAD, 1 mM DTT, 5 mM KNO_3, and 20 mM EDTA. The reaction was started by adding 200 µl of the extract and terminated after 5 min by adding 125 µl of the zinc acetate solution (0.5 M). The nitrite formed was measured colorimetrically by adding 750 µl of 1% sulfanilamide in 3 M HCl, and 750 µl of 0.02% N-naphtylethylenediamine hydrochloride, and an absorption was determined at 546 nm.

The activity of glutamine synthetase (GS, EC 6.3.1.2) was determined by means of the synthetase reaction (Elliot 1953). Briefly, the volume of the reaction mixture with 20 µM hydroxylamine and 100 µM Glu was 2.2 ml, including 0.5 ml of enzyme extract. Hydroxamate formation was measured in an assay mixture after 15 min at 30°C. The absorbance was measured at 540 nm.

N content and N accumulation: N concentration in plant tissue was determined by the standard macro-Kjeldahl procedure (*Nitrogen Analysis System*, *Büchi*, Flawil, Switzerland) in 7-day intervals after application of GLA. All dried leaf samples were ground in a Wiley mill to a size small enough to pass through a screen with 1-mm openings, mixed thoroughly, then subsampled for N determinations. The fine-ground sample of 0.1 g, 5 ml H_2SO_4, 0.23 g K_2SO_4, and 0.07 g $CuSO_4$ was placed into Kjeldahl flasks, then digested. The ammonia was distilled from an alkaline medium and absorbed in boric acid. The ammonia was determined colorimetrically by 0.020 M H_2SO_4 solution. The N accumulation was calculated as the product of the dry mass and N content.

Biomass and Chl content were measured at the same day as the N content (three plants in each treatment). Plants were dried at 80°C to constant mass and then weighed. Chl was extracted with absolute alcohol and acetone and measured using a spectrophotometer (*UV-2102C*, *Unico*, China), and Chl content was calculated according to Arnon's method (1949).

Data analysis: Results were analyzed by *SPSS* (*10.0 for Windows*, Chicago, IL, USA). Effects of GLA and time were analyzed using a two-way *ANOVA*. When significant according to the *ANOVA*, the comparisons between treatments were conducted with *Duncan*'s multiple range test for mean significances ($p<0.05$).

Results

The effect of exogenous GLA on the photosynthetic physiology parameters: Application of GLA improved the AQY (Table 1). The highest AQY was found in GLA_{800}, and there was a significant difference between it and control. The CE in GLA_{800} treatment was higher than in other treatments, which was 2.2-fold of GLA_{400}, 2.5-fold of GLA_{200} and 2.8-fold of control, and had a significant difference ($p<0.05$). The V_{cmax} was increased by 77.5%, 62.2%, and 11.5% of control value for GLA_{800}, GLA_{400}, and GLA_{200}, respectively. There was a significant difference among GLA_{800}, GLA_{400}, and control ($p<0.05$). The L_s in GLA_{800} and GLA_{400} were higher than in GLA_{200} and control and had a significant difference ($p<0.05$) (Table 1).

Gas exchange: There was an increase in P_N from 0 d to 14 d under GLA treatments, and then a decrease after 14 d (Fig. 1*A*). Application of GLA significantly increased P_N which was higher in GLA_{800} than in other treatments and control. The P_N reached the highest

Table 1. The effects of exogenous glutamic acid (GLA) on carboxylation efficiency (CE), apparent quantum yield (AQY), stomatal limitation value (L_s), and maximum carboxylation velocity of Rubisco (V_{cmax}) in hawthorn. Measured at 7 d after application of glutamic acid. The data are expressed as mean ± SE, $n = 7$, $p<0.05$.

GLA [mg l^{-1}]	CE [mol(CO_2) m^{-2} s^{-1}]	AQY [µmol(CO_2) $µmol^{-1}$(photon)]	L_s	V_{cmax} [µmol m^{-2} s^{-1}]
0	0.017 ± 0.003^b	0.012 ± 0.002^c	0.347 ± 3.0^b	24.4 ± 1.65^c
200	0.019 ± 0.004^b	0.019 ± 0.002^b	0.296 ± 1.8^c	27.2 ± 3.25^c
400	0.021 ± 0.003^b	0.019 ± 0.005^b	0.429 ± 1.5^a	39.6 ± 3.51^b
800	0.047 ± 0.005^a	0.023 ± 0.003^a	0.446 ± 2.7^a	43.3 ± 2.62^a

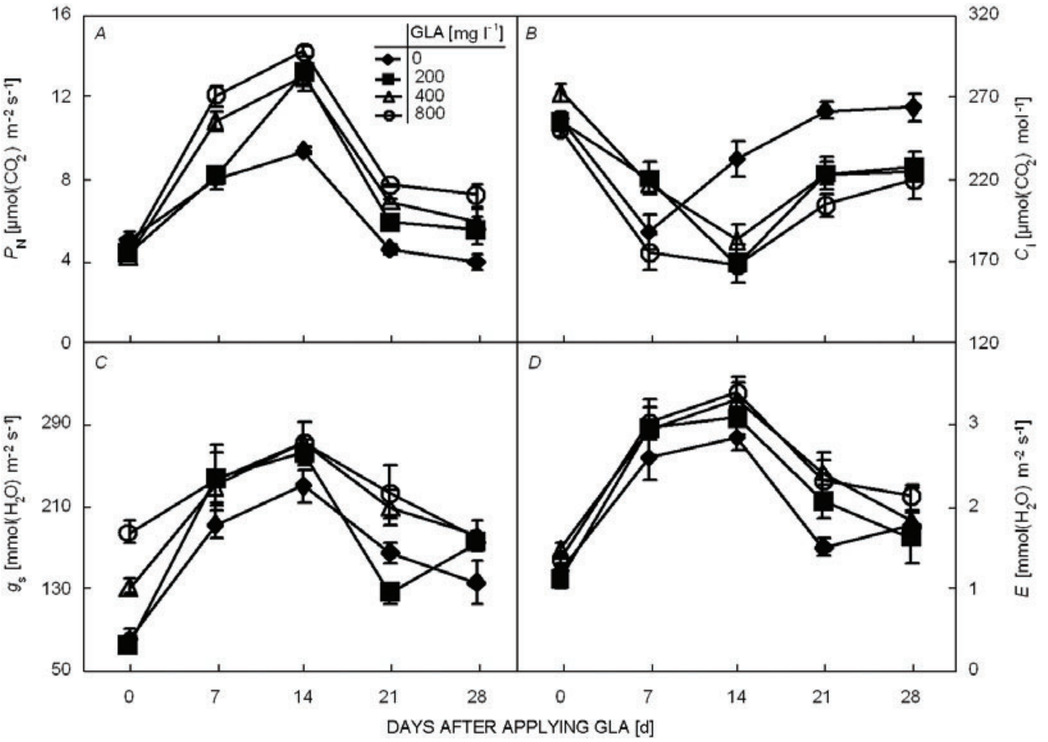

Fig. 1. Changes of *A*: net photosynthetic rate (P_N), *B*: intercellular CO_2 concentration (C_i), *C*: stomatal conductance (g_s), and *D*: transpiration rate (E) of hawthorn in different exogenous glutamic acid (GLA) treatments. *Bars* indicate SE ($n = 7$). Means are shown.

value at 14 d after application of GLA, and was increased by 51.9%, 38.2%, and 40.2% in GLA_{800}, GLA_{400}, and GLA_{200}, respectively, and had significant differences ($p<0.05$). Changes in g_s and E of all treatments were similar to P_N (Fig. 1*C*,*D*), which was the highest value at 14 d after application of GLA. The value of g_s in GLA_{800} was higher and had a significant difference compared to the control ($p<0.05$), while the changes in C_i were just contrary to those of P_N, g_s, and E (Fig. 1*B*). Application of GLA had no significant affects on C_i between days of 0 to 14, however, there was a significant difference after 14 d between GLA_{800} and control ($p<0.05$).

Chl fluorescence parameters: The F_m, F_v/F_m, F_v/F_0, Φ_{Eo} as well as PI_{ABS} increased after application of GLA, and reached the highest value at 14 d, while the minimal fluorescence (F_0) decreased and reached the lowest value at 14 d (Fig. 2). The values of F_v/F_m, F_v/F_0, Φ_{Eo} as well as PI_{ABS} in different concentrations of GLA treatments were higher than the control and had a significant difference (except at 0 d and 28 d) ($p<0.05$), while there were no significant differences between different concentrations of GLA ($p<0.05$).

NR and GS activities: NR is the key enzyme of nitrogen assimilation and affected the utilization of nitrogen. The value of NR activity showed an increasing trend and reached the highest value at 7 d after application of GLA, and then a decrease after 7 d (Fig. 3*A*). Application of GLA improved the NR activity compared to the control, among of which GLA_{800} was the best and had a significant difference compared to the control (except at 0 d and 28 d). The trend of change of GS activity was paralleled with NR (Fig. 3*B*). Application of GLA improved the GS activity compared to the control and had significant differences among GLA_{800}, GLA_{400}, and control at 7 d after application of GLA.

Soluble protein content: The soluble protein content increased gradually after application of GLA and reached the highest value at 21 d after treatments, and then decreased (Fig. 4). There was a significant difference between GLA_{800} and control from 14 d to 28 d after application of GLA ($p<0.05$). This result indicated that GLA supply accelerated the soluble protein accumulation.

Leaf N content and N accumulation: The leaf N content was the highest at 21 d after application of GLA, and had significant differences between GLA treatments and control (Fig. 5*A*). Application of GLA_{800} increased the N content compared to the control and had a significant difference ($p<0.05$) at 21 d. The N accumulation increased with the increasing of treatment days (Fig. 5*B*). Application of different concentrations of GLA always had higher N accumulation compared to the control and had a significant difference (except at 7 d) ($p<0.05$). However, it was higher in GLA_{800} than in other treatments and control (except at 28 d).

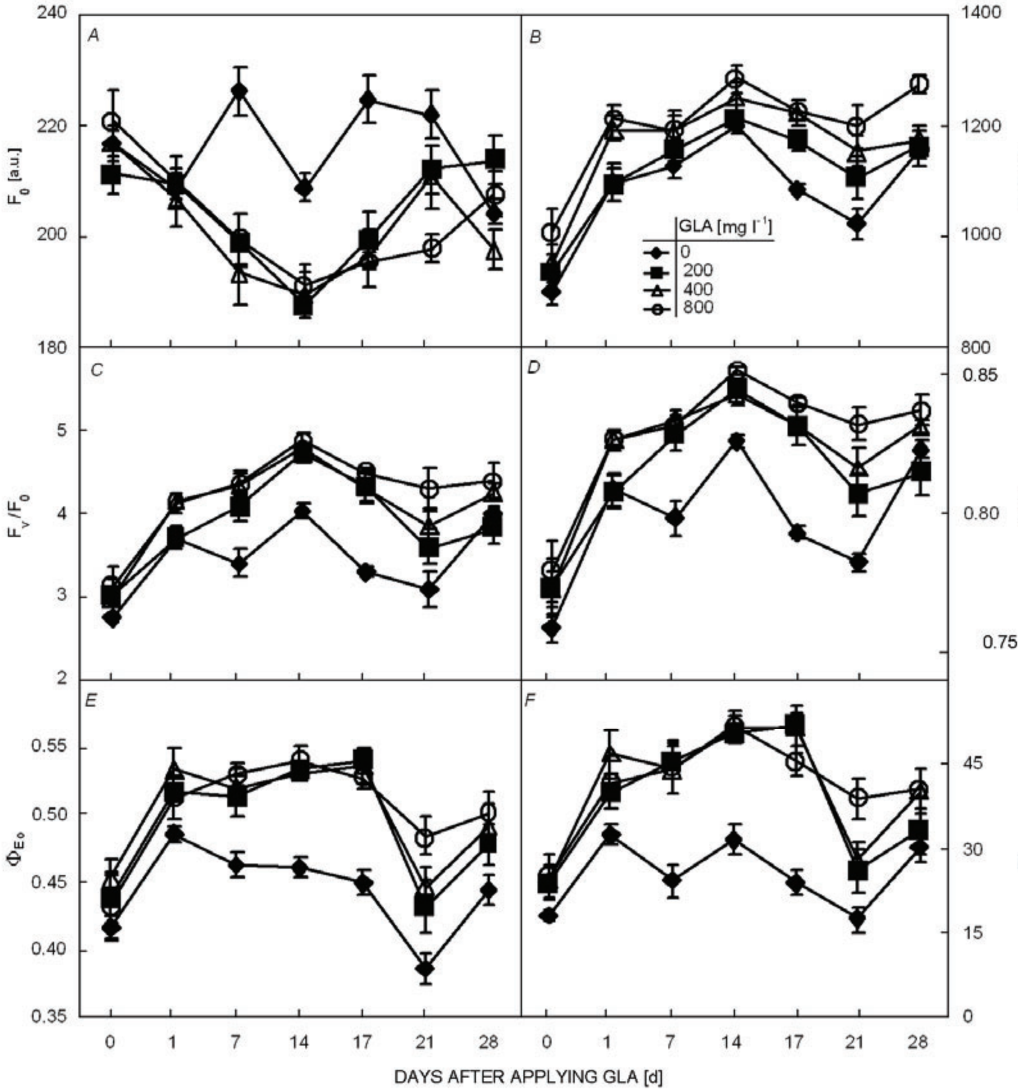

Fig. 2. Changes of *A*: the maximal fluorescence (F_m), *B*: the minimal fluorescence of dark adapted state (F_0), *C*: maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PSII (F_v/F_0), *D*: the maximal quantum yield of PSII (F_v/F_m), *E*: the probability that an absorbed photon will move an electron into the electron transport chain beyond Q_A (Φ_{Eo}), and *F*: performance index on absorption basis (PI_{ABS}) of hawthorn in different exogenous glutamic acid (GLA) treatments. *Bars* indicate SE (n = 7). Means are shown.

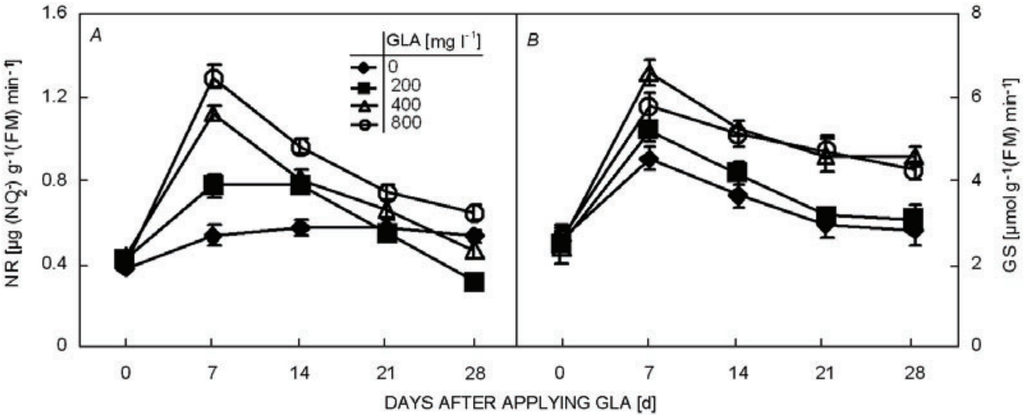

Fig. 3. Changes of *A*: nitrate reductase (NR) and *B*: glutamine synthetase (GS) activities of hawthorn in different exogenous glutamic acid (GLA) treatments. *Bars* indicate SE (n = 4). Means are shown.

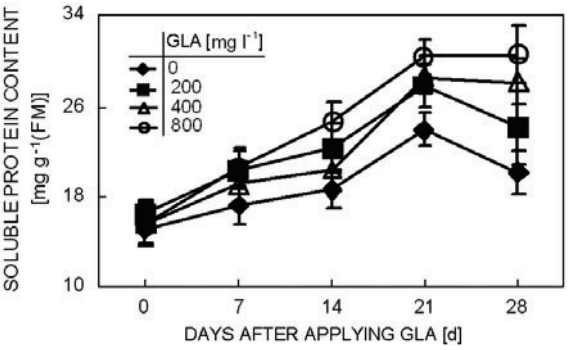

Fig. 4. Changes of soluble protein content of hawthorn leaves in different exogenous glutamic acid (GLA) treatments. *Bars* indicate SE ($n = 4$). Means are shown.

Chl content: Application of GLA induced an increase in Chl *a* and Chl *b* content between days of 0 to 14, and then a decrease (Fig. 6). Different concentrations of GLA had a different effects on the Chl content which was higher in GLA_{800} and had a significant difference compared to the control ($p<0.05$) (except at 0 d and 28 d).

The whole plant biomass increased with the increasing of treatment days and GLA concentrations (Fig. 7). The biomass in GLA_{800} was the highest and had a significant difference at 7 d after application of GLA compared to the control.

Discussion

Many researchers have found that the nitrogen content in a leaf exhibited strong positive relation with the P_N (Kao and Tsai 1999, Pal *et al.* 2005, Katahata *et al.* 2007, Evans, 1989, 1996). In this experiment, the P_N, Chl fluorescence, NR and GS activities, N content and N accumulation as well as the soluble protein content increased after application of GLA. Our results confirmed early reports that showed an increase in photosynthesis and Chl fluorescence parameter except F_0 (Kao and Tsai 1999, Pal *et al.* 2005, Wu *et al.* 2006), activities of NR (Pal *et al.* 2005) and GS, as well as in leaf N content (Aroar *et al.* 2001, Cai *et al.* 2008) when plants were subjected to application of nitrogen nutrients.

Normally, the increase of P_N induced by the increase of N level was caused by the increased g_s or carboxylation capacity. The present results indicated that exogenous GLA treatments not only led to an increase in g_s (Fig. 1*C*), but also a decrease in C_i (Fig. 1*B*), which was associated with an increase in P_N and F_v/F_m (Fig. 2*D*) as well as F_v/F_0 (Fig. 2*C*), implicating that nonstomatal limitation to photosynthesis at application of GLA may occur. Additionally, we can infer that the increase of g_s with the increase of GLA concentrations didn't play the main role in the increase of P_N, while the enhancement of energy capture of antenna pigments due to the increase of chlorophyll content, the increase of F_v/F_m, Φ_{Eo} and PI_{ABS} was probably another reason for P_N increasing.

The two key enzymes involved in assimilation of intracellular ammonium into organic compounds are NR and GS, which also participate in photosynthetic and carbohydrate metabolism (Lam *et al.* 1996, Sibout and Guerrier 1998). Although suitable N level enhances photosynthesis and delays senescence (Wu *et al.* 2008, Guo *et al.* 2007, Sinclair *et al.* 2000), generally, N deficiency results in a decrease in leaf N content (Sinclair *et al.* 2000, Llorens *et al.* 2003, Zhao *et al.* 2003), and NR and GS activities (Sibout and Guerrier 1998, Abd-El Baki *et al.* 2000). This is important because NR and GS can be associated with amino acid conversion (Lam *et al.* 1996), and amino acid composition might be altered due to the application of GLA (Suzuki and Knaff 2005). The results suggest that the key enzymes of nitrogen metabolism play an important role in the increase of P_N and plant growth when GLA was applied.

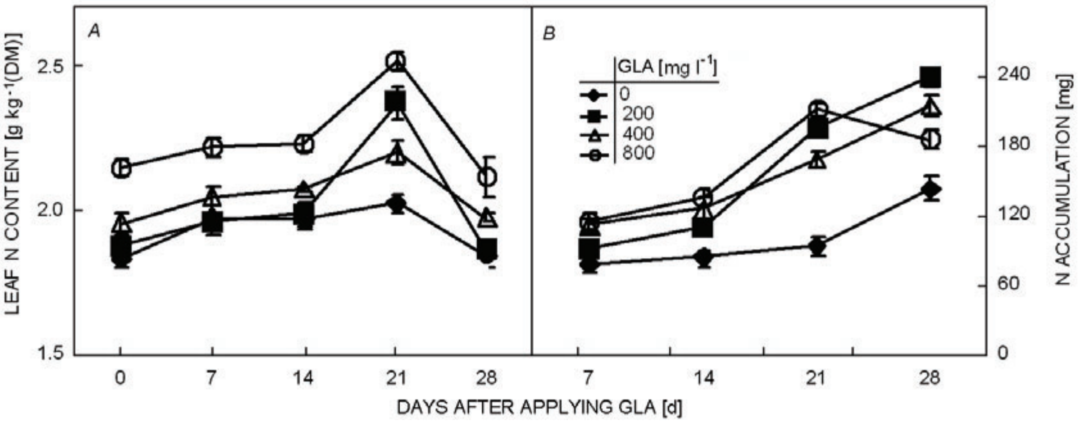

Fig. 5. Changes of nitrogen (N) content (*A*) and N accumulation (*B*) of hawthorn in different exogenous glutamic acid (GLA) treatments. *Bars* indicate SE ($n = 4$).

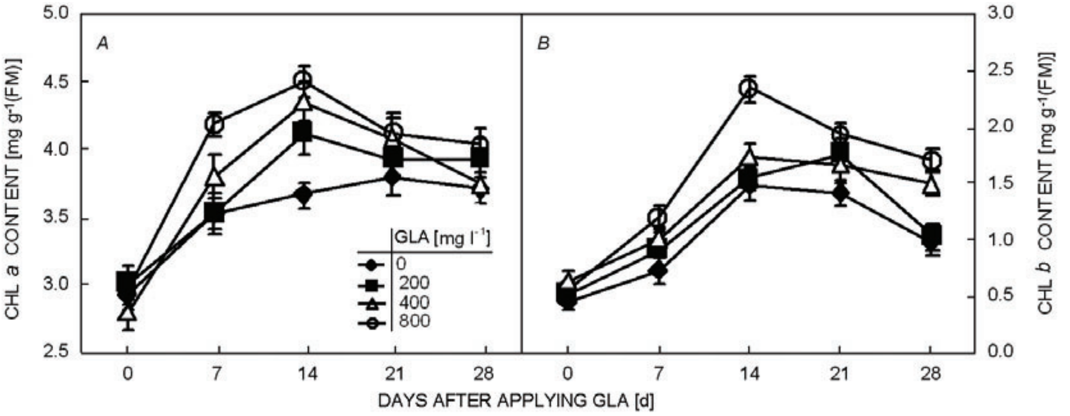

Fig. 6. Changes of chlorophyll (Chl) *a* (*A*) and Chl *b* (*B*) contents of hawthorn in different exogenous glutamic acid (GLA) treatments. *Bars* indicate SE ($n = 4$).

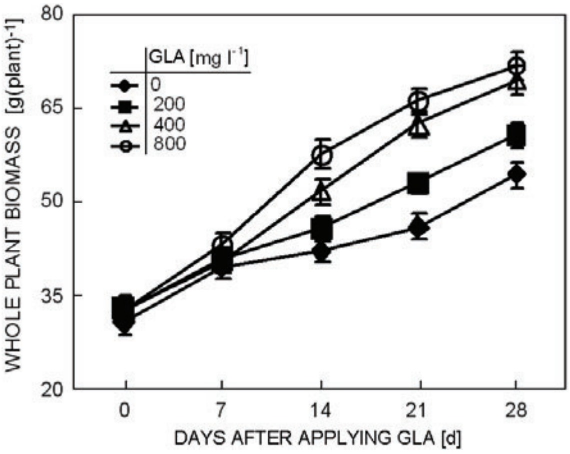

Fig. 7. Changes of whole plant biomass of hawthorn in different exogenous glutamic acid (GLA) treatments. *Bars* indicate SE ($n = 4$).

Photosynthetic capacity is closely associated with leaf N (Mae 1997, Llorens *et al.* 2003). Leaf N content is important because it is associated with the CO_2 assimilatory capacity of crops (Hikosaka 2004). P_N and V_{cmax} increase with increasing leaf N (Fig. 1*A*, Table 1). Thus, the relative content of leaf N can be used to estimate of photosynthetic capacity (Evans 1983, Llorens *et al.* 2003, Takashima *et al.* 2004). In elevated-CO_2 studies, plants under N deficit also show low photosynthetic capacity (Deckmyn and Impens 1997, Lima *et al.* 1999, Ainsworth *et al.* 2003, Bondada and Syvertsen 2005), suggesting that leaf N plays a key role in photosynthetic acclimation to environmental variables. Leaf N increased gradually with time, and with the increase of GLA concentrations (Fig. 5*A*), agreeing with reports by Warren *et al.* (2003). This increase in leaf N is closely associated with leaf development and photosynthetic capacity (Mae 1997, Llorens *et al.* 2003), which can be related to improved components of the photosynthetic apparatus, such as chloroplast thylakoid membranes (Kolodziejek *et al.* 2003). In addition, the increase in soluble protein content was also observed when GLA was supplied, especially at GLA_{800} (Fig. 4), which showed that the 1,5-diphosphoribulose carboxylase and carboxylation capacity were increased. Soluble protein concentration could serve as an index for Rubisco protein and activity (Pal *et al.* 2005), because mean Rubisco protein concentrations in plant leaves comprise around 30–50% of total soluble protein (Sicher and Bunce 1997, Long *et al.* 2006, Stitt and Schulze 1994).

Application of amino acids may act by diverting photosynthetically fixed carbon from synthesis of primary metabolites such as cellulose, lignin, lipids, and proteins that are associated with growth metabolism to synthesis of secondary metabolites like flavonoids and other. This is the process by which application of exogenous amino acids changes the synthetic ways of secondary metabolites, and needs further study to clarify the effect of amino acids on the regulatory mechanism of secondary metabolites.

The present results indicate that application of GLA accentuated the positive effects of nitrogen metabolism by regulating the activities of key enzymes involved in nitrogen assimilation and catabolism, and led to the increase of photosynthetic capacity and soluble protein content, suggesting that the key enzymes of nitrogen metabolism would play a key role in the increase of photosynthetic assimilation of plants after application of GLA, particularly at GLA_{800}. Thus, appropriate GLA supply is recommended for hawthorn to improve photosynthetic processes and nitrogen metabolism, and indirectly contributes to regulation of secondary metabolisms.

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