Effect of 5-aminolevulinic acid on photosynthesis, yield, nutrition and medicinal values of kudzu (*Pueraria phaseoloides*)

FENG XU[1], JUN ZHU[2], SHUIYUAN CHENG[1,3], WEIWEI ZHANG[1] AND YAN WANG[1]
[1] College of Horticulture and Gardening, Yangtze University, Jingzhou, China
[2] Department of Bio-medicine, Zhongshan Torch Polytechnic, Zhongshan, China
[3] College of Life Science and Engineering, Huanggang Normal University, Huanggang, China

Abstract

Kudzu (*Pueraria phaseoloides*) is an ecologically and economically important forage legume and cover crop in tropical and subtropical regions. The effects of foliar application of different concentrations (30, 100 and 300 mg/L) of 5-aminolevulinic acid (ALA) on photosynthesis, growth, chemical composition and medicinal components of seedlings of kudzu were tested in a pot experiment. Seedlings were sprayed twice weekly for 3 weeks and parameters measured at 8 weeks after the initial application. Treatment with ALA at 100 mg/L significantly increased DM yield of total plants plus chlorophyll concentration, photosynthetic rate and stomatal conductivity of kudzu leaves and reduced CO_2 concentration in the leaf, but had no significant effect on transpiration rate. Application of ALA (100 mg/L) significantly increased nitrogen, phosphorus, potassium and calcium concentrations in leaves as well as the soluble sugar, starch and vitamin C concentrations. Furthermore, the accumulation of medicinal components including flavonoids and puerarin was also significantly increased by ALA application. While this study suggests that ALA application could promote yield and quality of kudzu, further work is needed to determine optimal application rates and treatment regimes before recommendations could be made on the commercial application of this technology.

Correspondence: Feng Xu and Shui Yuan Cheng, College of Horticulture and Gardening, Yangtze University, Jingzhou 434025, China. E-mail: xufeng198@126.com or s_y_cheng@sina.com

Introduction

The forage legume kudzu (*Pueraria phaseoloides*) is widely distributed and frequently introduced into production systems or gardens around the world. It has become an important pasture crop in some tropical countries because of its ability to improve soil fertility and the nutritional quality of pastures by its nitrogen-fixing capability (Halim 1992). The high protein concentration in kudzu makes it an excellent supplement for animal feeding (Hussain *et al*. 1989), increasing both milk and meat production of cattle (Dirven 1965). In addition, roots of kudzu are used widely in Chinese traditional medicine. The main active constituents of *Pueraria* roots, including puerarin and isoflavonoids, have hypothermic, spasmolytic, hypotensive and antiarrhythmic qualities (Si *et al*. 2006). Work is currently underway to improve the nutritional and medicinal values of kudzu by increasing yield.

The keto-amino acid, 5-aminolevulinic acid (ALA), with a molecular weight of 131, is a precursor of heme, chlorophyll, vitamin B_{12} and other tetrapyrrole compounds *in vivo* (Stobart and Ameen-Bukhari 1984). ALA serves as prosthetic groups of respiratory enzymes, and chlorophyll in bacteria (Brunham and Lascelles 1963) and plants (Granick 1961), and is the major photosynthetic light-harvesting pigment (Senge 1993). Low concentrations (10–300 mg/L) of ALA enhanced growth rates and photosynthesis, when applied at the 3–4 leaf stage to barley, potato, radish, garlic and kidney bean (Hotta *et al*. 1997a), resulting in increased crop yields. ALA at 10 and 100 mg/L presumably promoted chilling tolerance of melon seedlings at low light intensity, reflected in improved chlorophyll biosynthesis and photosynthetic activity and suppression of respiration (Hotta *et al*. 1997b; Wang *et al*. 2004). Recently,

Watanabe *et al*. (2006) found that 100 mg/L of ALA enhanced the growth and photosynthesis of grapevines, while Hotta *et al*. (1998) and Zhang *et al*. (2006) showed that ALA increased cold and salt tolerance in rice and potato. This compound therefore appears to act as a hormone-like plant growth regulator, which is effective at relatively low concentrations (10–100 mg/L). Furthermore, ALA has low mammalian toxicity and is also biodegradable (Kennedy *et al*. 1990).

However, there have been few reports of application of ALA to pastures. As such, the objective of this study was to investigate the effects of ALA application on photosynthesis, yield and nutritional and medicinal values of kudzu.

Materials and methods

Plant materials and ALA treatments

Kudzu seeds were sterilised with 0.1% sodium hypochlorite for 3–5 min, washed 3 times with water and placed in a Petri dish containing a double thickness of filter paper. The seeds were soaked in water and incubated in a plastic container at 30°C for 3 days. The germinated seeds were sown in pots filled with a sterilised soil mixture (soil:vermiculite:manure 1:1:0.2) and the experimental seedlings were selected after 20 days on the basis of vigorous and uniform vegetative growth. The selected seedlings were transplanted into pots (25 cm diameter, 30 cm height) (1 plant/pot) filled with sandy soil (sand:humus soil 1:2). Row spacing was 2 m. All seedlings were placed in a growth chamber with 12 h photoperiod (24/24°C, day/night; 450 μmol/m^2/s) at 70% RH. Five hundred ml of liquid fertiliser (containing 0.36 g N, 0.12 g P and 0.14 g K) was applied weekly to each pot. After the third leaves were fully expanded, seedlings were sprayed with aqueous solutions of ALA (Sigma, St Louis, MO, USA; ALA dissolved in acetic acid buffer solution, pH 4.6) at 3 concentrations (30, 100 and 300 mg/L), while the control seedlings (0 mg/L ALA) were sprayed with the acetic acid buffer (pH 4.6). Twenty ml of solution was applied twice/week in the morning for 3 weeks. Six replications (9 plants/replication) were used per treatment and samples pooled for each determination. Measurements were made 8 weeks after the initial application of ALA.

Determination of chlorophyll

Fully expanded leaves (100 g) were ground in a mortar (adding liquid nitrogen) and then homogenised in cold (4°C) 80% v/v acetone in water. The homogenate was kept in the dark and centrifuged at 10 000 rpm for 5 min to remove the leaf debris. The absorbance of the extract at 647 and 664 nm was measured using a spectrophotometer (DU 730, Beckman Coulter Inc., USA). For the accurate determination of chlorophyll a, chlorophyll b and the total (chlorophyll a + b), the extinction coefficients of Graan and Ort (1984) were used.

Measurements of growth and photosynthesis

Photosynthetic rate, transpiration rate and stomatal conductivity as an index of the stomatal aperture, and CO_2 concentration in the leaf were measured by using a LiCor-6400 portable photosynthesis system (LiCor, USA) 8 weeks after the initial application of ALA. Photosynthetic rate, transpiration rate and stomatal conductivity were measured on the fifth leaf after 2 h of acclimation in a growth cabinet, at a temperature of 24°C under a light intensity of 1000 μmol/m^2/s and relative humidity of 60%, and replicated 6 times (9 plants/replication). The plants were then harvested for determination of biomass. The dry weights of leaf, shoot and root were determined after oven-drying fresh samples (80°C) for 48 h.

Chemical analysis

Total N concentration was measured by the indophenol method (Sagi 1966) and P concentration by the molybdovanadophosphate colorimetric procedure (Apostolatos 1984). The concentrations of potassium (K), calcium (Ca), magnesium (Mg) and iron (Fe) were measured by an atomic absorption spectrophotometer (AI-1200, Aurora Instruments Co. Ltd, Canada).

Samples were analysed for total soluble sugar, starch and vitamin C following the methods of Zhang *et al*. (2006), Letchworth and Lambert (1998) and Zhang *et al*. (2006), respectively.

Puerarin was extracted from samples of leaf and shoot and determined following the HPLC method of Kintzios *et al*. (2004), which was detected at 248 nm. Total flavonoids of shoot and

leaf samples were extracted and measured following the method of Cheng *et al*. (2004).

Statistical analysis

All data obtained were subjected to analysis of variance (ANOVA) using the SPSS version 10.0 statistical package for Windows (SPSS 1999). Mean separation was carried out using Duncan's Multiple Range Test at the 5% probability level.

Results

Yields of all components (leaves, shoots and roots) and photosynthetic rates of leaves increased significantly up to 100 mg/L ALA then declined at 300 mg/L ($P<0.05$) (Tables 1 and 2). CO_2 concentration in leaf declined significantly from 0 to 100 mg/L ALA and then increased to 300 mg/L (Table 2). While transpiration rate was not significantly affected by ALA application, stomatal conductivity was significantly higher ($P<0.05$) at 100 and 300 mg/L than at 0 and 30 mg/L.

Concentrations of chlorophyll a and chlorophyll b and chlorophyll a + b increased from 0 to 100 mg/L ALA and then declined to 300 mg/L (Table 3). Compared with 0 mg/L ALA, plants treated with ALA showed large and significant increases in N, P, K and Ca concentrations, with peak responses usually occurring at 100 mg/L ALA (Table 4). However, ALA application had no significant effect on the concentration of Mg and little effect on Fe concentration.

Table 1. Effects of 5-aminolevulinic acid (ALA) on dry weights of roots, shoots and leaves of kudzu seedlings over 8 weeks.

Parameter	ALA concentration (mg/L)			
	0	30	100	300
Weight of roots (g)	15.86c[1]	18.35b	19.19a	16.14c
Weight of shoots (g)	39.46b	39.80b	46.92a	40.63b
Weight of leaves (g)	4.28b	4.59ab	5.88a	4.71ab
Weight of total plant (g)	59.60b	62.74b	71.99a	61.48b

[1] Means within rows followed by different letters are significantly different ($P<0.05$).

Table 2. Effects of 5-aminolevulinic acid (ALA) on photosynthetic and transpiration rates, stomatal conductivity and CO_2 concentration in the leaves of kudzu seedlings.

Parameter	ALA concentration (mg/L)			
	0	30	100	300
Photosynthetic rate (μmol/m^2/s)	10.43c[1]	11.75b	13.05a	11.22b
Transpiration rate (mmol/m^2/s)	4.37a	4.26a	4.94a	4.75a
Stomatal conductivity (mmol/m^2/s)	316.3b	324.6b	367.5a	352.1a
CO_2 concentration (μL/L)	187.3a	182.1ab	164.6b	181.5ab

[1]Means within rows followed by different letters are significantly different ($P<0.05$).

Table 3. Effects of 5-aminolevulinic acid (ALA) on chlorophyll (Chl) concentration in the leaves of kudzu seedlings.

Parameter	ALA concentration (mg/L)			
	0	30	100	300
Chl a (mg/g FW)	0.84c[1]	0.92b	1.27a	0.85c
Chl b (mg/g FW)	0.35b	0.42ab	0.50a	0.32b
Chl a+b (mg/g FW)	1.19c	1.34b	1.77a	1.17c

[1]Means within rows followed by different letters are significantly different ($P<0.05$).

Table 4. Effects of 5-aminolevulinic acid (ALA) on N, P, K, Ca, Mg and Fe concentrations in kudzu seedlings (including roots, shoots and leaves).

Parameter	ALA concentration (mg/L)			
	0	30	100	300
Total N (mg/g)	2.76c[1]	3.17b	3.37a	3.05b
Total P (mg/g)	3.05b	3.42ab	3.65a	3.60a
Total K (mg/g)	4.31b	4.40b	4.96ab	5.21a
Total Ca (mg/g)	0.78c	0.86b	0.95a	0.84b
Total Mg (mg/g)	0.41a	0.43a	0.40a	0.45a
Total Fe (µg/g)	67.26a	61.20b	67.33a	68.04a

[1]Means within rows followed by different letters are significantly different (P<0.05).

Table 5. Effects of 5-aminolevulinic acid (ALA) on soluble sugar, starch and vitamin C concentrations in the leaves of kudzu seedlings.

Parameter	ALA concentration (mg/L)			
	0	30	100	300
Soluble sugars (mg/g FW)	7.63b[1]	8.71a	8.79a	7.59b
Starch (% DM)	2.31b	2.78a	2.80a	2.76a
Vitamin C (µg/g FW)	59.25b	57.96b	68.14a	60.02b

[1]Means within rows followed by different letters are significantly different (P<0.05).

Table 6. Effects of 5-aminolevulinic acid (ALA) on puerarin and flavonoids in shoots and leaves of kudzu seedlings.

Parameter	ALA concentration (mg/L)			
	0	30	100	300
Leaf				
Puerarin (% DM)	0.17c[1]	0.19b	0.21a	0.16c
Flavonoids (% DM)	1.73b	1.96ab	2.07a	1.82b
Shoot				
Puerarin (% DM)	2.25b	2.48ab	2.82a	2.78a
Flavonoids (% DM)	3.90b	4.72a	4.75a	3.93b

[1]Means within rows followed by different letters are significantly different (P<0.05).

Soluble sugar concentrations were significantly (P<0.05) higher at 30 and 100 mg/L ALA than at 0 and 300 mg/L, while starch concentrations were significantly (P<0.05) higher at 30, 100 and 300 mg/L ALA than in controls (Table 5). Vitamin C concentration was significantly higher at 100 mg/L than on the remaining treatments (Table 5).

ALA treatment increased the puerarin and flavonoid concentrations in both leaves and shoots (Table 6) with peak responses usually occurring at 100 mg/L ALA.

Discussion

This study has provided interesting results on the positive impact of spraying with low concentrations of ALA on growth and chemical composition of kudzu. This agrees with the findings of Hotta *et al*. (1997a) and Watanabe *et al*. (2006) that the yields of plants, including barley, garlic, kidney bean, potato, radish and grape, could be improved by 10–60% by ALA foliar treatment at low concentration (30–300 mg/L). These authors assumed that the higher production was related to an increase in photosynthetic rate and CO_2 fix-

ation and reduced release of CO_2 in darkness. Our observations on kudzu seedlings support this assumption and show that application of low levels of ALA increased leaf chlorophyll concentration, photosynthetic rate and stomatal conductivity.

Not only did application of ALA increase growth, but also rates of 30–100 mg/L significantly increased concentrations of N, P, K and Ca in plant shoots and leaves, indicating a dramatic increase in uptake of these nutrients by kudzu plants. Increased N concentration following ALA treatment has also been reported in spinach (Yoshida *et al*. 1995), while Watanabe *et al*. (2006) reported significant increases in N, K and Ca concentrations in grapevine when ALA was applied. These increases in mineral nutrients could result from an increase in the nitrate reductase activity (Mishra and Srivastava 1983), although the mechanisms by which ALA accomplishes this are unclear. A more detailed examination is required. If this practice was to be applied in the future, applications of these nutrients in fertiliser would need to be increased to prevent soil rundown.

The increases in soluble sugar, starch and vitamin C concentrations of kudzu have significant implications for use of the forage produced. In particular, vitamin C is an essential nutrient for healthy body function and is also important as an antioxidant and radical scavenger in plants (Smirnoff 2000). It has been suggested that ALA increases fructan concentration (Yoshida *et al*. 1995) and that the effects of ALA appear to be related to the transfer and storage of carbohydrates and also to the formation of polysaccharides (Bingshan *et al*. 1998).

It has been proposed that ALA could promote the accumulation of the products involved in phenylpropanoid metabolism in plants, such as anthocyanin in apples (Wang *et al*. 2004; Wang *et al*. 2006). The marked increases in flavonoid and puerarin concentrations in leaves and shoots of kudzu in our study following ALA treatment should greatly enhance the medicinal value of kudzu plant material. In unpublished studies, we have showed that ALA greatly increased the concentrations of total flavonoids and anthocyanins and enhanced the activity of flavonoid pathway enzymes such as phenylalanine ammonia-lyase, chalcone synthase and chalcone isomerase in *Ginkgo biloba* leaves. We concluded that enhanced flavonoid accumulation was a result of increased activity of enzymes involved in flavonoid biosynthesis. We suggest that the effects of ALA on the activity of enzymes involved in flavonoid and puerarin biosynthesis should be investigated further.

Conclusion

This study was conducted under ideal conditions in a glasshouse. While results suggest that application of ALA at low concentrations can increase growth of kudzu through increased photosynthetic rate, with concomitant increases in N, P, K and Ca concentrations, how this information might be used commercially still needs to be resolved. The optimal concentration to use and the ideal treatment frequency are not obvious from this study. Before recommendations could be made on application of this technology by farmers, these aspects need to be resolved and carry-over effects of treatment following 8 weeks of age need to be assessed.

Acknowledgements

This work was supported by the Program for New Century Excellent Talents in University (NCET-04-0746), the Youth Talent Foundation of Hubei Province (2003ABB014), the Educational Office Key Research Program of Hubei Province (Z200627002) of China and the Doctor Foundation of Yangtze University (0010113).

References

Apostolatos, G. (1984) Technical note: a rapid procedure for determination of phosphorus from a Kjeldahl digest. *International Journal of Food Science and Technology*, **19**, 643–646.

Bingshan, L., Hotta, Y., Yinglan, Q., Jingsong, Z., Tanaka, T., Takeuchi, Y. and Konnai, M. (1998) Effects of 5-aminolevulinic acid on the growth and ripening of wheat. *Journal of Pesticide Science*, **23**, 300–303.

Brunham, B.F. and Lascelles J. (1963) Control of porphyrin biosynthesis through a negative-feedback mechanism. Studies with preparations of 5-aminolevulate synthetase and B-ALA dehydratase from *Rhodopseudomonas spheroids*. *Biochemical Journal*, **87**, 462–472.

Cheng, S., Wang, Y., Fei, Y. and Zhu, G. (2004) Studies on the effects of different treatments on flavonoids contents in *Ginkgo biloba* leaves and their regulating mechanism. *Journal of Fruit Science*, **21**, 116–119.

Dirven, J.G.P. (1965) Chemical composition and nutritive value of tropical kudzu (*Pueraria phaseoloides* (Roxb.) Benth). *Plant Foods for Human Nutrition*, **12**, 185–198.

Graan, T. and Ort, D.R. (1984) Quantitation of the rapid electron donors to P 700, the functional plastoquinone pool and the ratio of the photosystems in spinach chloroplast. *The Journal of Biological Chemistry*, **259**, 14003–14010.

Granick, S. (1961) Magnesium protoporphyrin monoester and protoporphyrin monomethyl ester in chlorophyll biosynthesis. *Journal of Biology and Chemistry*, **236,** 1168–1172.

Halim, R.A. (1992) *Pueraria phaseoloides* (Roxb.) Benth. In: Mannetje, L. 't and Jones, R.M. (eds) *Plant Resources of South-East Asia No. 4, – Forages*. pp. 192–196. (Pudoc Scientific Publishers: Wageningen, The Netherlands).

Hotta, Y., Tanaka, T., Takaoka, H., Takeuchi, Y. and Konnai, M. (1997a) Promotive effects of 5-aminolevulinic acid on the yield of several crops. *Plant Growth Regulation*, **22**, 109–114.

Hotta, Y., Tanaka, T., Takaoka, H. and Takeuchi, Y. (1997b) New physiological effects of 5-aminolevulinic acid in plants: the increase of photosynthesis, chlorophyll content, and plant growth. *Bioscience, Biotechnology, and Biochemistry*, **61**, 2025–2028.

Hotta, Y., Tanaka, T., Bingshan, L., Takeuchi, Y. and Konnai, M. (1998) Improvement of cold resistance in rice seedlings by 5-aminolevulinic acid. *Journal of Pesticide Science*, **23**, 29–33.

Hussain, A., Bushuk, W. and Roca, W.M. (1989) Identification of cultivars of the forage legume *Pueraria phaseoloides* by electrophoretic patterns of storage proteins. *Euphytica*, **41**, 71–73.

Kennedy, J.C., Pottier, R.C. and Pross, D.C. (1990) Photodynamic therapy with endogenous protoporphyrin IX: basic principle and present clinical experience. *Journal of Photochemistry and Photobiology: Biology*, **6**, 143–148.

Kintzios, S., Makri, O., Pistola, E., Matakiadis, T., Shi, H.P. and Economou, A. (2004) Scale-up production of puerarin from hairy roots of *Pueraria phaseoloides* in an airlift bioreactor. *Biotechnology Letters*, **26**, 1057–1059.

Letchworth, M.B. and Lambert, R.J. (1998) Pollen parent effects on oil, protein, and starch concentration in maize kernels. *Crop Science*, **38**, 363–367.

Mishra, S.N. and Srivastava, H.S. (1983) Stimulation of nitrate reductase activity by delta aminolevulinic acid in excised maize leaves. *Cellular and Molecular Life Sciences*, **39**, 1118–1120.

Sagi, T. (1966) Determination of ammonia in sea water by the indophenol method and its application to the coastal and offshore waters. *The Oceanographical Magazine*, **18**, 43–51.

Senge, M.O. (1993) Recent advances in the biosynthesis and chemistry of chlorophylls. *Photochemistry and Phytobiology*, **57**, 189–206.

Si, J., Chang, Q., Shen, L. and Chen, D. (2006) Chemical constituents from *Pueraria lolbata*. *Journal of Chinese Pharmaceutical Sciences*, **15**, 248–250.

Smirnoff, N. (2000) Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Current Opinion in Plant Biology*, **3**, 229–235.

SPSS (1999) *SPSS 10.0.1 for Windows*. (SPSS Inc.: Chicago, Illinois).

Stobart, A.K. and Ameen-Bukhari, J. (1984) Regulation of δ-aminolevulinic acid synthesis and protochlorophyllide regeneration in the leaves of dark-grown barley (*Hordeum vulgare*) seedlings. *Biochemical Journal*, **222**, 419–426.

Wang, L.J., Wang, Z.H., Li, Z.Q., Liu, H., Liu, W.Q., Chen, Z.Y., Yan, P. and Sun, D.Q. (2004) Effect of 5-aminolevulinic acid on enhancing apple fruit coloration. *Journal of Fruit Science*, **21**, 512–515.

Wang, Z., Tang, G., Li, Z. and Wang, L. (2006) Promotion of 5-aminolevulinic acid and genistein on anthocyanin accumulation in apples. *Acta Horticulturae Sinica*, **33**, 1055–1058.

Watanabe, K., Nishihara, E., Watanabe, S., Tanaka, T., Takahashi, K. and Takeuchi, Y. (2006) Enhancement of growth and fruit maturity in 2-year-old grapevines cv. Delaware by 5-aminolevulinic acid. *Plant Growth Regulation*, **49**, 35–42.

Yoshida, R., Tanaka, T. and Hotta, Y. (1995) Physiological effects of 5-aminolevulinic acid in vegetable crops. *Abstract of the 15th International Conference on Plant Growth Substances*, *Minneapolis, USA*. ***32*****,** 417.

Zhang, Z.J., Li, H.Z., Zhou, W.J., Takeuchi, Y. and Yoneyama, K. (2006) Effect of 5-aminolevulinic acid on development and salt tolerance of potato (*Solanum tuberosum* L.) microtubers *in vitro*. *Plant Growth Regulation*, **49**, 27–34.

(*Received for publication April 14, 2010; accepted July 15, 2010*)