Effect of Abiotic and Biotic Elicitors on Growth and Alkaloid Accumulation of *Lycoris chinensis* Seedlings

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Z. Naturforsch. **64c**, 541–550 (2009); received December 30, 2008/March 6, 2009

Three-month-old seedlings of *Lycoris chinensis* were treated with biotic and abiotic elicitors: yeast elicitor (YE), methyl jasmonate (MJ), salicylic acid (SA), and sodium nitroprusside as NO donator (NO). We have shown that the addition of MJ and NO promotes the accumulation of galanthamine in these seedlings. The effect of these elicitors on the growth of the seedlings, as well as on the amount of the alkaloids accumulated in the seedlings was studied. The results showed that, in general, high doses of MJ and SA had a negative effect on the growth of the seedlings, while appropriate doses of NO and YE had a positive effect on the growth of the seedlings. It was remarkable that the addition of MJ, NO, and YE can promote galanthamine accumulation in seedlings. The accumulation was higher in treatments at higher concentrations of NO (100 μM), where the release of galanthamine was 1.72-fold higher than that of the control at the 10th day of culture. The highest values of lycorine were obtained in seedlings treated with YE at a concentration of 0.01 g/l and by the 10th day of culture; the level was 1.38 times of the control.

Key words: Lycoris chinensis, Galanthamine, Alkaloid Accumulation

Introduction

Species of *Lycori*s have been planted as ornamentals in China and Japan for many centuries because they are diverse in colour and shape. They are well known also for producing structurally unique alkaloids that have a wide range of interesting physiological effects such as antitumour, antiviral, acetylcholinesterase-inhibitory, immunostimulatory, and antimalarial activities (Bastida *et al.*, 1987). Because galanthamine (GAL) is an important drug for the symptomatic treatment of senile dementia or Alzheimer's disease (AD), it is the most newly promising AChE inhibitor – in the USA by the Food and Drug Administration and in Europe by the European Registration Bureau (Howes *et al.*, 2003; Laurain-Mattar, 2008). For many years a lot of research effort was spent to synthesize GAL (Czollner *et al.*, 1998; Parsons *et al.*, 2001; Herlem *et al.*, 2003; Küenburg *et al.*, 1999), but the total organic synthesis of GAL is complicated and thus expensive. Meanwhile much research has been done to find new ways to produce GAL by biological technology, for example by adding exogenous growth regulators during organogenesis and somatic embryogenesis (Colque *et al.*, 2004) and transformation of *Leucojum aestivum* L. using *Agrobacterium rhizogenes* to form hairy root cultures (Diop *et al.*, 2007). Despite this wild *Lycoris* plants remain the most important source of GAL, although the production of this metabolite and the availability of the plant are limited. One species, *Lycoris chinensis* (Amaryllidaceae), is uniquely distributed in Southern China. Although the GAL content of this species is only up to 0.6‰ of the fresh weight (FW) of bulbs, it has greater growth potential than other species. This species also has the ability to accumulate the related alkaloids lycorine and lycoramine.

Plants interact with their environment by releasing a diverse array of secondary metabolites, that include alkaloids. As secondary metabolite,

Abbreviations: DW, dry weight; FW, fresh weight; GA3, gibberellic acid; GAL, galanthamine; MJ, methyl jasmonate; NO, nitric oxide; SA, salicylic acid; SNP, sodium nitroprusside; YE, yeast elicitor.

0939–5075/2009/0700–0541 $ 06.00

GAL may play a critical role when the plant is subjected to biotic and abiotic stresses that may modulate the alkaloid biosynthesis and accumulation. At present one of the used techniques to promote product accumulation in cultured seedlings is the addition of biotic and abiotic elicitors. Despite many of them are of microbial origin, certain compounds, such as mineral salts and various organic compounds, have been recognized for their capability to trigger the accumulation of secondary metabolites. Thus, some of the compound responses are triggered by abiotic and biotic stresses, such as methyl jasmonate (MJ) (Clérivet and Alami, 1999; Conceição *et al.*, 2006; Cho *et al.*, 2008), salicylic acid (SA) (Cho *et al.*, 2008), and yeast elicitor (YE) (Ge and Wu, 2005). In the present study we also tested if nitric oxide (NO) can stimulate GAL accumulation, because it has been reported to be included in responses to abiotic and biotic stresses, such as drought, salt, and heat stresses, disease resistance, and apoptosis (Delledonne *et al.*, 1998; Duner and Klessig, 1999; Zhao *et al.*, 2007). Whether it can improve the Amaryllidaceae-type alkaloids accumulation such as that of GAL has not been demonstrated.

In the present study the effects of MJ, SA, NO and YE on both growth and alkaloid production of three-month-old seedlings of *Lycoris chinensis* were investigated. The objective of this investigation was performed primarily to increase the production of GAL, but also the concentration of total alkaloids including that of two related

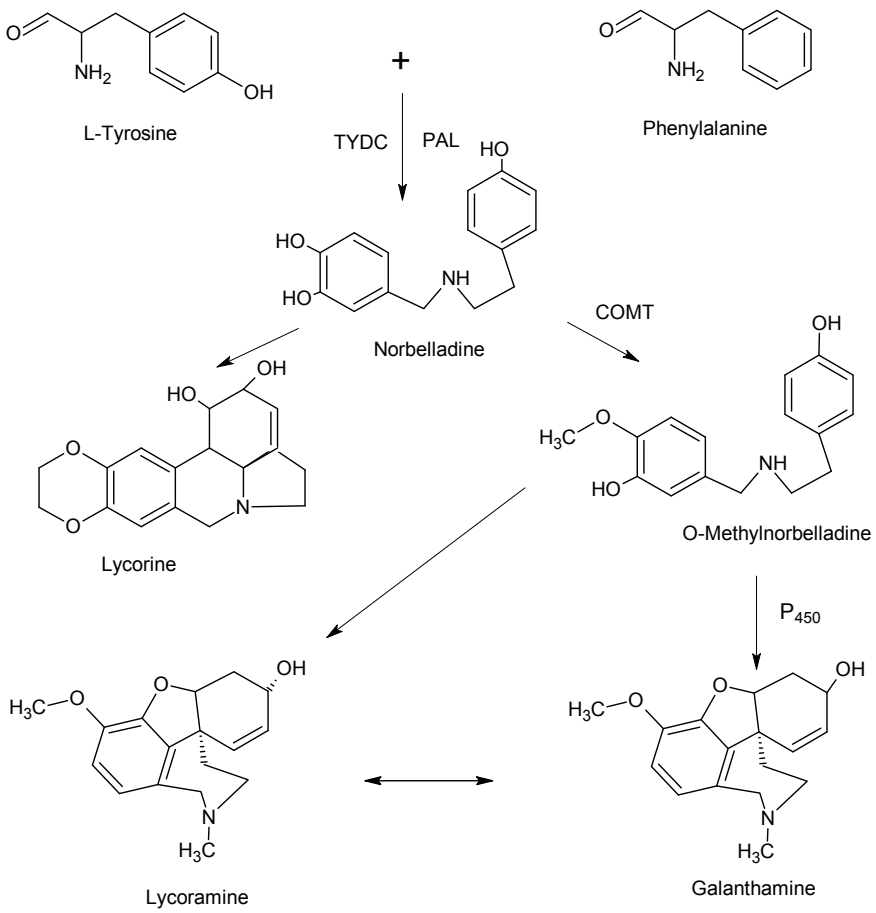

Fig. 1. Biosynthetic pathway and relationships of alkaloids from *Lycoris chinensis* (Battersby *et al.*, 1964; Mann *et al.*, 1963; Eichhorn *et al.*, 1998). Enzyme abbreviations: TYDC, tyrosine-decarboxylase; PAL, phenylalanine-ammonia-lyase; COMT, catechol-*O*-methyltransferase; P450, cytochrome P450 enzyme.

alkaloids, lycorine and lycoramine. Lycorine is a toxic crystalline alkaloid. It inhibits protein synthesis, and may inhibit the ascorbic acid biosynthesis, although studies on the latter are controversial and inconclusive. Lycoramine (1,2-dihydrogalanthamine), another GAL-type alkaloid, has been claimed to have significant activities in inhibiting the formation of the peptide bond in protein synthesis (Han *et al*., 1992). The biosynthetic pathway and relationships among these compounds are shown in Fig. 1.

Experimental

Plant material and treatments

All experiments were performed using *Lycoris chinensis*. Field-grown plants were raised in an experimental field at Jiangsu Province Key Laboratory for Medicinal Plant, Nanjing, China. In November 2007, mature seeds were collected and stored at 4 ºC. After one month, the seed capsules were peeled off, and the seeds were sterilized by incubation in 75% (v/v) ethanol for 1 min followed by incubation in 0.1% mercury dichloride (corrosive mercuric chloride) for 10 min. Afterwards, the seeds were washed eight times with sterile distilled water before placing them onto tissue culture medium containing Murashige and Skoog (MS) salts, at pH 5.8, solidified with agar (7.5 g/l). During this experiment all the media were autoclaved for 15 min at 121 ºC. The seeds were cultured in 6 cm diameter "baby-food jars" containing 25 ml of MS solid medium supplemented with 30 g/l of sucrose and 0.3 mg/l of giberellic acid GA3, which has been proved by our previous experiments to be optimal for seed germination. After culture for three months, the seedlings were transplanted to media containing different concentrations of MJ, SA, SNP (sodium nitroprusside), and YE. The accumulation of the alkaloids in the seedling was subsequently determined. The seedlings increase was showed as growth index (GI) determined by the formula: GI = (final weight – initial weight)/initial weight.

Preparation of elicitors

The YE was the carbohydrate (polysaccharide) fraction of the yeast extract prepared by ethanol precipitation, as described by Hahn and Albersheim (1978). Concisely, 25 g of YE (Oxoid Ltd., Baingstoke, Hampshire, England) were dissolved in 125 ml of distilled water; then 100 ml of ethanol were added. The solution was permitted to precipitate for 4 d at 4 ºC in a refrigerator, and the supernatant was discarded. The remaining glutinous deposit was redissolved in 125 ml of distilled water and subjected to another round of ethanol precipitation. The final deposit was dissolved in 100 ml of distilled water, sterilized by autoclaving at 121 ºC for 2 h, and stored at 4 ºC in a refrigerator before use. The elicitor concentration was expressed by the total carbohydrate content, which was determined by the phenol/sulfuric acid method (Dubois *et al*., 1956) using sucrose as a standard.

Elicitor treatments

All the substances except for YE used as elicitors in this experiment were purchased from Sigma. The different concentrations used were established according to Table I (the YE dose was expressed by the total carbohydrate content, which was determined by the phenol/sulfuric acid method using sucrose as a standard). Since these doses were very different for each substance, we introduced the same numbers (from 1 to 4) for each treatment to figure the four doses (from the lowest to the highest, respectively), thus making distincter the presentation of the results. Thus, for instance, treatments SNP1, SNP2, SNP3 and

Table I. Elicitors used at different treatment concentrations.

Elicitor	Treatment 1	Treatment 2	Treatment 3	Treatment 4
MJ [μM]	20	50	100	250
SA [mM]	0.1	0.25	0.5	1.0
SNP [μM]	5	50	100	500
YE[a] [g/l]	0.01	0.05	0.10	0.15

[a] The yeast elicitor dose is expressed by the total carbohydrate content, which was determined by the phenol/sulfuric acid method using sucrose as a standard.

SNP4 figured doses of 5, 50, 100, and 500 μM of SNP apart. MJ and SNP were dissolved in absolute ethanol and distilled water, respectively, and then sterilized through a sterile filter of 0.22 μm pore diameter, YE was prepared according to the method depicted above, and SA was dissolved in distilled water. SA and YE were added directly to the culture media, which were then autoclaved, whereas MJ and SNP were added after the media had been autoclaved. Because ethanol was used to dissolve MJ, the same quantity of ethanol was added to the other treatments, involving the control. After treatment for 3, 10 and 25 d, the seedlings were collected and stored at –80 °C. The growth indexes of the seedlings were measured after treatment for 25 d.

Alkaloid extraction and analysis

Determination of GAL, lycorine and lycoramine was accomplished based on the method of Colque *et al.* (2004). The whole seedlings were dried in an oven at 45 °C until constant weight and then powdered with a mortar and pestle. 100 mg of powder were macerated with 10 ml of methanol for 24 h at room temperature; during this time the mixture was sonicated thrice at regular intervals (30 min each). The methanolic extracts were centrifuged at 4000 rpm for 20 min, and then fil-

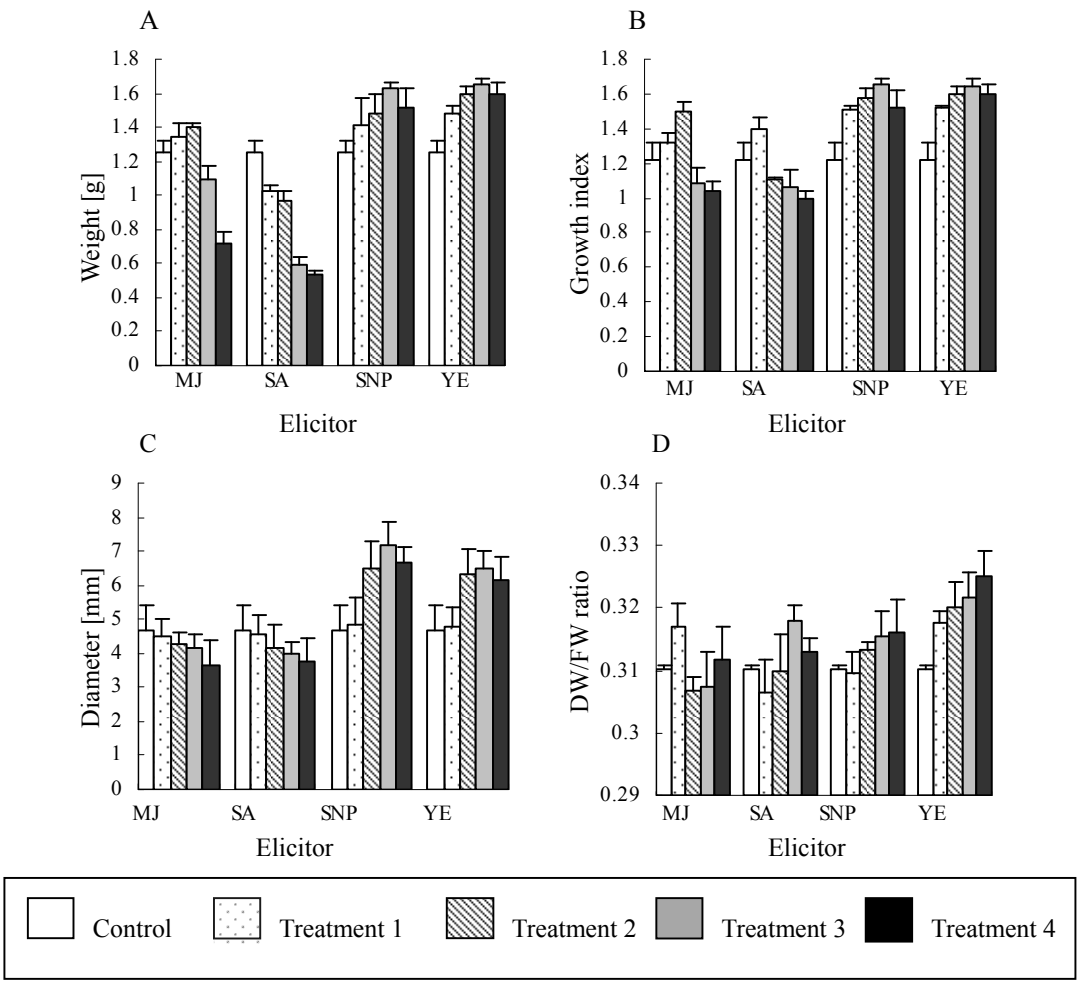

Fig. 2. Effect of the elicitors on (A) weight, (B) growth index (GI), (C) diameter, and (D) DW/FW ratio of the *Lycoris chinensis* seedlings. Values represent the means of five replicates ± standard deviation.

tered through a 0.22-μM pore filter (Millipore) before HPLC analysis. The quantitative amounts of the alkaloids lycorine, GAL and lycoramine were determined by HPLC analysis as described by Li *et al.* (2003). The standards of GAL, lycorine and lycoramine were purchased from Fujian Like Bio-pharmaceutical Technology Co., Ltd. (Batch No. 061210–2, purity ≥ 98.0%).

Statistical analysis

The results belonging to the growth index of the seedlings and alkaloid production of the seedlings represent the average of five replicates. The data were subjected to statistical analysis of variance, with $p < 0.05$. In the graphs, columns and lines represent means and error bars represent standard deviations.

Results

Effect on growth

The effect of the elicitors on the growth of seedlings was different depending on the elicitor compound and its concentration. Thus, SA at any dose and MJ at higher concentration restrained the growth of the seedlings (Fig. 2). SNP applied at

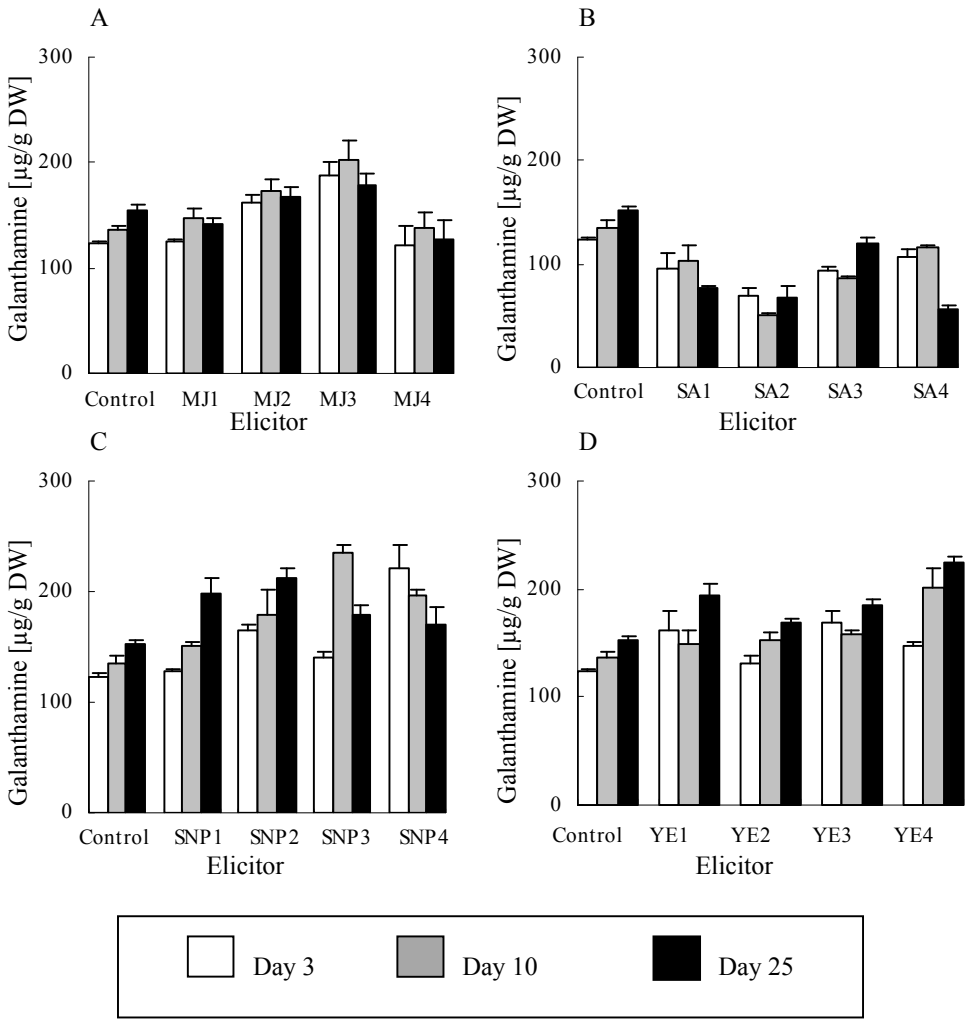

Fig. 3. Levels of GAL accumulated in the seedlings of *Lycoris chinensis* treated with different elicitors. Values represent the means of five replicates ± standard deviation.

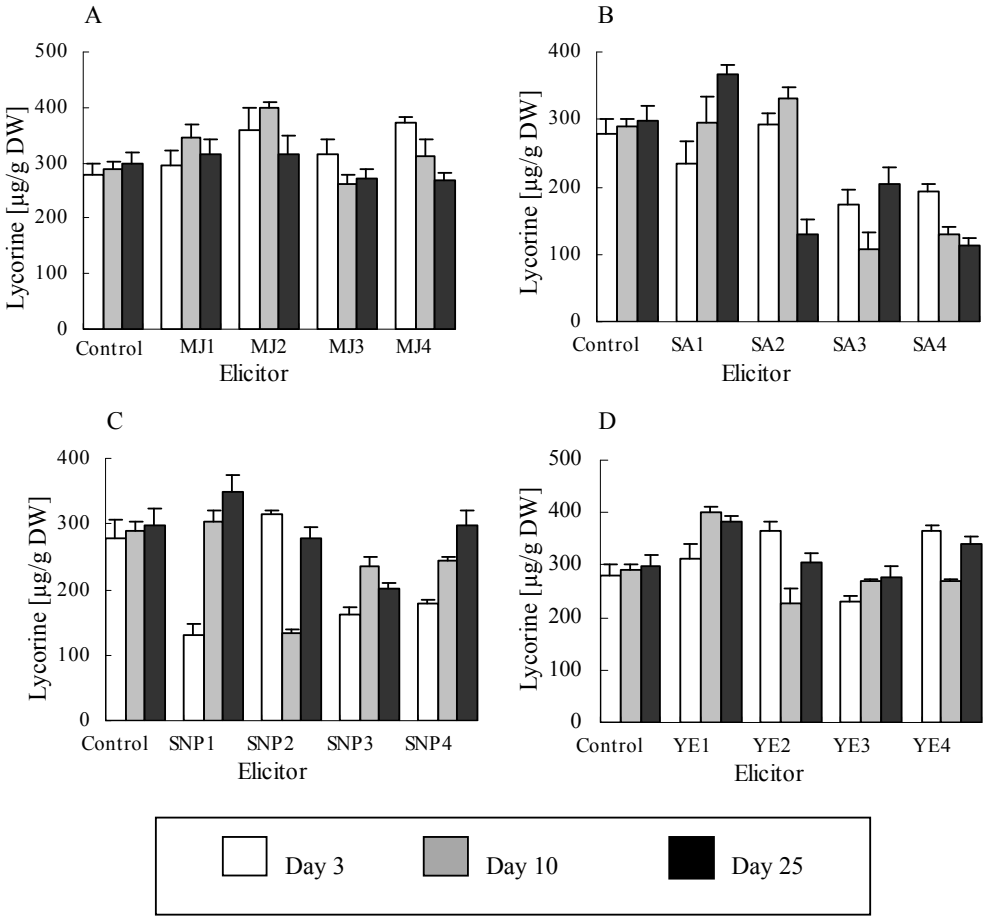

Fig. 4. Levels of lycorine accumulated in *Lycoris chinensis* seedlings treated with different elicitors. Values represent the means of five replicates ± standard deviation.

intermediate concentrations (50, 100 and 500 μM) and YE applied at appropriate concentrations (0.05, 0.1 and 0.15 g/l) significantly increased the growth of the explants with respect to the control. However, the lowest concentrations of SNP and YE used did not affect the growth of the seedlings. Compared to the growth index, no obvious differences in the DW/FW ratio were observed between the seedlings treated with the different organic compounds and the control (Fig. 2), which showed that all the seedlings accumulated similar contents of dry matter.

GAL production

Fig. 3 shows the resulting levels of GAL accumulated in the seedlings at the end of the experiments. It is notable that the addition of MJ, SNP, YE could enhance the amount of GAL accumulated in the seedlings. This effect was more accentuated in the treatment with the higher concentration of SNP3 (100 μM), where the accumulation of GAL was 1.72-fold higher than that of the control at the 10th day of culture, meanwhile this increase was approx. 1.15- and 1.18-fold higher at day 3 and day 10 of the assay. GAL was also accumulated at higher levels in YE4 (0.15 g/l) elicited cultures, approx 1.62 times greater than the control one, whereas this increase was about 1.20-fold and 1.48-fold higher at days 3 and 10 of the assay.

MJ at the three higher concentrations also increased the accumulation of GAL in the seedlings compared with the control, though the dif-

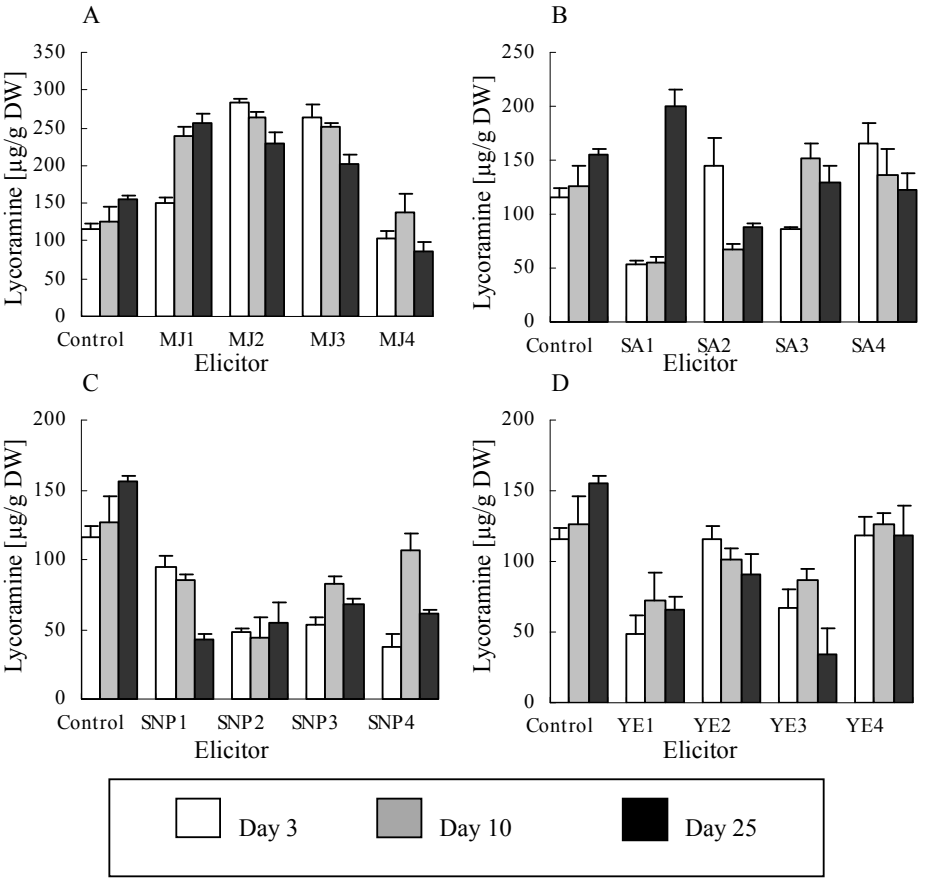

Fig. 5. Levels of lycoramine accumulated in the seedlings of *Lycoris chinensis* treated with different elicitors. Values represent the means of five replicates ± standard deviation.

ferences were not statistically significant (Fig. 3). This effect was more notable with the treatment at higher concentrations of MJ3 (100 μM), where the accumulation of GAL was 1.49-fold higher than that of the control at the 10th day of culture. By contrary, the addition of SA in the culture medium restrained the accumulation of GAL in the seedlings in all the applied concentrations (Fig. 3).

Production of GAL-related alkaloids

The results concerning the production of individual alkaloids other than GAL are shown in Fig. 4 and 5. Lycorine was produced in higher quantities compared to the other individual alkaloids. The accumulation of lycorine was stimulated mainly by MJ and YE (Fig. 4). The highest level of lycorine was obtained in the seedlings treated with 0.01 g/l YE at the 10th day of culture which was 1.38 times of control seedlings; MJ at 50 μM (MJ2, day 10) also remarkably increased the accumulation of lycorine in the seedlings with respect to the control, which was 1.37 times that of the control. SNP1 (5 μM) at the 25th day of culture and SA1 (0.1 mM) at the 25th day of culture also increased the accumulation of lycorine (Fig. 4), although not notably different from the control seedlings.

The amount of lycoramine accumulated in the seedlings was also triggered by MJ, although contrarily with the concentration of the elicitor (Fig. 5). On the contrary, the presence of SA, SNP and YE in the culture medium inhibited the accumulation of lycoramine in the seedlings in all the used doses.

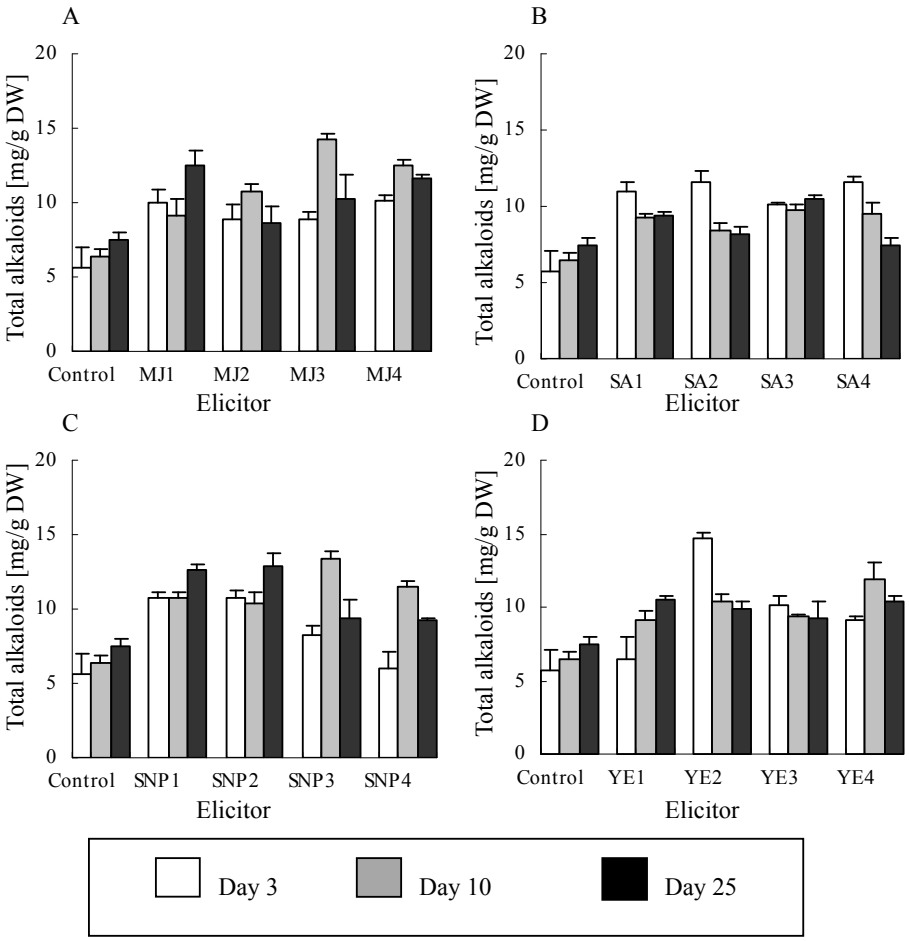

Fig. 6. Levels of total alkaloids accumulated in the seedlings of *Lycoris chinensis* treated with different elicitors. Values represent the means of five replicates ± standard deviation.

Total alkaloids production

The levels of the total alkaloids accumulated in the seedlings are shown in Fig. 6. The presence of MJ, SA, SNP and YE in all the concentrations applied significantly increased the accumulation of the total alkaloids. This effect was more pronounced at the dose of 0.05 g/l YE (YE2), where the amount of alkaloids accumulated in the seedlings after the 3rd day of culture was 2.56-fold higher than that observed for the control seedlings, respectively.

Conclusion

The outline of the alkaloids accumulated in the seedlings varied throughout the experiment. The addition of MJ significantly favoured the accumulation of GAL, lycorine, lycoramine and total alkaloids in the seedlings, whereas the addition of YE significantly favoured the accumulation of GAL, lycorine and total alkaloids in the seedlings. Contrastingly the addition of SNP significantly favoured the accumulation of GAL and total alkaloids in the seedlings, while the addition of SA did not have any effect on the production of the Amaryllidaceae-type alkaloids, and at the same time SA also improved the accumulation of total alkaloids in the seedlings. It is noteworthy that, as a whole, the amounts of alkaloids accumulated by the control seedlings followed the order: lycorine > GAL > lycoramine.

Table II. Galanthamine content of different materials under different culture conditions.

Researcher	Species	Extract	Culture condition[a]	Galanthamine (10^{-3} % DW)
Sellés *et al.*, 1997	*Narcissus confusus*	Seed-derived explants	2,4-D (1 mg/l) + BA (5 mg/l)	61
Sellés *et al.*, 1999	*Narcissus confusus*	Dedifferentiated calli	2,4-D (2 or 4 mg/l) or picloram (4 mg/l)	0.003
		Embryogenic callus	BA (1 mg/l)	0.011
		Shoot clumps	2,4-D (1 mg/l) + BA (5 mg/l)	0.014
		Plantlets	2,4-D (1 mg/l) + BA (5 mg/l)	0.143
Colque *et al.*, 2004	*Narcissus confusus*	Shoot clumps	BA (3 mg/l) + PPM (0.5 ml/l) + MJ (25 μM)	800
Diop *et al.*, 2006	*Leucojum aestivum*	*In vivo* bulbs	–	0
		Embryogenic calli	2,4-D (5 μM) + BA (5 μM)	0
		Embryogenic calli	2,4-D (25 μM) + BA (0.5 μM)	44.8
		Embryogenic calli	2,4-D (10 μM) + BA (10 μM)	73
Diop *et al.*, 2006	*Leucojum aestivum*	*In vivo* bulblets developed on hairy roots	Clone 1–5	10.3–51.3
Diop *et al.*, 2007	*Leucojum aestivum*	Bulbs *in vitro* cultures	Picloram (10 μM) + BA (0.5 μM)	0
		In vitro bulblets	Without growth regulator	1.14
		In vitro bulblets	NAA (10 μM) + BA (0.5 μM)	6.79
		In vitro bulblets	NAA (0.5 μM) + BA (5 μM)	4.74
		In vitro roots	NAA (10 μM) + BA (0.5 μM)	0
Present study	*Lycoris chinensis*	Three-month-old seedlings	YE (0.15 g/l)	25.1

[a] BA, benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α-naphthalene acetic acid; PPM, plant preservative mixture.

The present study showed that GAL accumulation and accumulation of related alkaloids in seedlings of *Lycoris chinensis* can be stimulated by both biotic and abiotic elicitors. The amount of total alkaloids, GAL, lycorine, and lycoramine accumulated in the seedlings was improved by MJ with respect to the control. The rules of the GAL accumulation in the seedlings in this study are similar to results of Colque *et al.* (2004). They found that the GAL content in the tissues was increased maximal by 2.00 times (from approx. 4.0 mg/g DW to about 8.0 mg/g DW) when MJ (50 μM) was added. In the present study, GAL accumulation in the seedlings was 1.49-fold higher than in the control (from about 135.0 μg/g DW to about 203.0 μg/g DW) when MJ (100 μM) was fed. The distinctness is due to the difference of the material.

In summary, the production of GAL by *Lycoris chinensis* seedlings cultured in MS medium with the addition of MJ, NO, YE resulted in an increased GAL accumulation in the seedlings. Comparing the GAL content in our experiment with that of other researchers (Table II), although the GAL content in *Lycoris chinensis* is not the highest, this species grows fast and is easy to seed. It is a promising material for breeding a new species with high GAL content and growth amount.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant no. 30700057) and the opening fund of Jiangsu Province Key Laboratory for Plant Ex-Situ Conservation (KF07001). We thank Dr. Evan Evans from Tasmanian Institute of Agricultural Research at University of Tasmania and Prof. Wenbiao Shen from College of Life Sciences at Nanjing Agricultural University for critical reading and linguistic help in the preparation of this manuscript.

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