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Full Length Research Paper

Effect of fungal elicitor on carbon and nitrogen status and triterpenoid production in cell suspension cultures of *Betula platyphylla* Suk.

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Carbon and nitrogen status, as well as triterpenoid production were investigated in cell suspension cultures of *Betula platyphylla* Suk. treated with fungal elicitor. The fungal elicitor was observed to inhibit the cell growth and enhanced triterpenoid production. The maximum triterpenoid production was 123.82 mg·L^{-1} DW in the 1-day elicitated cells, which was two fold higher than that of the control. The response of carbon and nitrogen status to the fungal elicitor was 18.62% decreased sucrose, 307.79% decreased soluble proteins, 146.93% increased total soluble sugar, 484.54% increased glucose, slightly increased total amino acids and 906.96% (about 9-fold) increased C/N ratio in the 1-day elicitation, which slowly equals the control with increasing incubation time. These results indicate that changes in carbon and nitrogen status were activated or were partially directed towards triterpenoid production, especially C/N ratio.

Key words: *Betula platyphylla* Suk., carbon, fungal elicitor, nitrogen, triterpenoid.

INTRODUCTION

Plant cell and organ culture systems offer a viable alternative for the production of commercial important secondary metabolites for the food and pharmaceutical industries (Sahai and Knuth, 1985; Rao and Ravishankar, 2002). One of the major pharmaceutical secondary metabolites, namely: triterpenoids, extracted from the bark of *Betula platyphylla* Suk., is an excellent drug with antiviral, antibacterial, antitumor, and anti-AIDS activity (Alakurtti et al., 2006; Chintharlapalli et al., 2007; Csuk et al., 2010). In a previous study, triterpenoids (betulin and oleanolic acid) were found in *B. platyphylla* Suk. cell cultures, but the content was significantly lower than that of the bark (Fan et al., 2009; Yin et al., 2009). To improve triterpenoid production, a *Phomopsis* fungal strain (CCTCC M 209271, http://www.cctcc.org) (Supplementary data; Figure 1) derived from the inner bark of *B. platyphylla* has been shown as an effective elicitor of triterpenoid synthesis in *B. platyphylla* cell cultures. The application of fungal elicitors are a well recognized tool for increasing the production of secondary metabolites in plant cell cultures (Farme, 1985; Cramer et al., 1985), and its elicitor components including cell wall fragments, polysaccharides, oligosaccharide, glycoprotein, etc. (Lu and Mei, 2003). Plant cells respond to fungal elicitor treatment by activating a wide variety of reactions, such as ion fluxes across plasma membranes, synthesis of reactive oxygen species, as well as phosphorylation and dephosphorylation of proteins, which have frequently been discussed as putative components of signal transduction chain(s), leading to elicitor-induced defense responses, such as the activation of defense genes, hypersensitive cell death, and systemic acquired resistance (Dietrich et al., 1990; Eder and Cosio, 1994; Menke et al., 1999; Zhao et al., 2001; Scott et al., 2001; Qiao et al., 2010). The principal basis for signal transduction of these defense responses is believed to involve nitric oxide through a jasmonic acid–dependent signaling pathway and, eventually, biosynthesis of secondary metabolites, such as pigments, flavones, terpenes, phytoalexins, and other defense-related compounds (Xu et al., 2005). Secondary metabolites are derived from primary metabolites such as carbohydrates

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Supplementary Figure 1. Regeneration of *Betula platyphylla* Suk. through tissue culture.

and amino acids. These primary metabolites are also essential nutritional ingredients for plant growth and development. However, the nutritional basis for elicitor signaling, leading to the stimulation of secondary metabolite production is still obscure. The objective of the present study is to investigate the responses of carbon (C) and nitrogen (N), major plant nutrients, to *Phomopsis* elicitors, and to comprehensively explain the relationship of C and N status with fungal elicitor–induced triterpenoid production in cell suspension cultures of *B. platyphylla* Suk. Besides, this study is useful for determining the feasibility of large-scale triterpenoid production using *B. platyphylla* Suk.

MATERIALS AND METHODS

Plant materials

The cell line used in the study was developed from the axillary buds of a 30-year-old *B. platyphylla* Suk. Suspension cultures were established from this cell line and cultivated on optimized Nagata–Takebe (NT) medium supplemented with 0.1 mg·L^{-1} 6-benzyladenine (6-BA), 0.01 mg·L^{-1} thidiazuron (TDZ), and 20 g·L^{-1}sucrose at an interval of 7 to 10 days. The pH of the medium was adjusted to 5.6 with 1 M NaOH before autoclaving. A previous report demonstrated considerable variation in the amount of triterpenoids with subcultures (Fan et al., 2010). For this reason, experiments were done in stabilized cultures. Cultures were considered stabilized when triterpenoid production was similar after four consecutive subcultures (Supplementary data; Figures 2 and 3). A single stock culture grown in a 1000 ml Erlenmeyer flask was used as inocula for the experimental flasks. All experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of the corresponding liquid media with 20 g·L^{-1} sucrose and inoculated with 4.0 g fresh weight of eight-day-old cell suspension cultures. The Erlenmeyer flasks were incubated on a rotary shake (110 rpm) at 25°C. Illumination was regulated to give 14 h of light (photophase 06.00 to 20.00 h) provided by fluorescent tubes (mixing Osram fluora and Osram daylight types) with a photon flux density (400 to 700 nm).

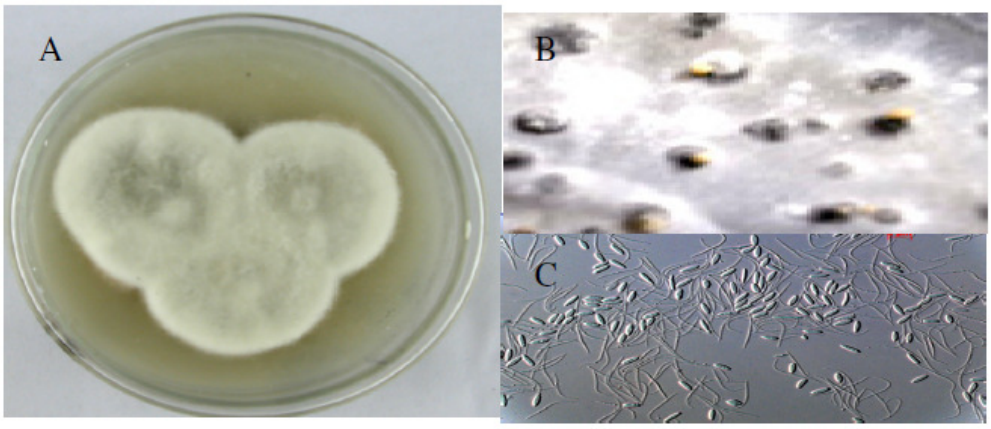
Fungal elicitor experiment

Endophytic fungi of *Phomopsis* was maintained on potato dextrose agar (PDA) medium consisting of glucose 20 g·L^{-1} at 4°C. Liquid cultures were initiated from seven-day-old PDA by inoculating 2 cm^{-2} agar cubes into 100 ml of PDA liquid medium. The flasks were incubated at 25°C for 10 days. The mycelia and culture solution were collected, and the elicitor was prepared according to the method described by Zhang et al. (2000). The elicitors were added at a polysaccharide concentration of 40 μg·L^{-1} in eight-day-old *B. platyphylla* Suk. cell cultures. The polysaccharide concentration of the elicitors was determined by the phenol-sulfuric acid method using glucose as the standard (Dubois et al., 1956) (Supplementary data; Figure 3).

Dry cell weight and triterpenoid estimation

For dry cell weight estimation, cells were harvested and collected by centrifugation at 3000 rpm for 15 min and washed with distilled water. The fresh cells were dried at 60 ± 2°C to a constant dry weight. Extraction of triterpenoids was done with 95% methanol. The extract was thereafter analyzed by ultraviolet spectrophotometer at a 510 nm wavelength using betulin as the

Supplementary Figure 2. The cell line of *Betula platyphylla* Suk.



Supplementary Figure 3. The colony morphology and spores photomicrographs of *Phomopsis.* Picture A is 7d colony morphology, B is one month colony morphology, C is photomicrographs of spores derived from orange overflow in *Phomopsis* colony.

standard (Fan et al., 2009).

Quantification of soluble sugars, soluble proteins and amino acids

Sucrose, glucose and fructose were extracted from 0.1 g dry cell suspension with 5 ml of 80% (v/v) ethanol, and assayed by colorimetric analysis using 480, 480 and 510 nm wavelengths (Lowry et al., 1951). Total soluble sugars were calculated as the sum of fructose, glucose and sucrose. Soluble proteins were measured with Coomassie blue reagent by spectrophotometer at 595 nm. Bovine albumin was used as the standard (Bradford, 1976). The amino acids content of the samples were analyzed and quantitatively determined using HPLC (Waters System Interface module 501, Hewlett–Packard, California, USA) as described by the Pico-tag method (Hainida et al., 2008). The C/N ratio is the ratio of the concentrations of soluble sugars to soluble proteins.

Statistical analysis

All experiments were repeated three times. The data obtained were statistically analyzed by SPSS (8.0). Means and standard errors

Table 1. The average date of 17 kinds of amino acids.

Days after treatment		Aspartate	Threonine	Serine	Glutamic acid	Glycine	Alanine	Cystine
1d	Treatment	1.52	0.70	1.00	8.63	0.73	1.74	0.33
	Control	1.54	0.70	1.00	9.08	0.72	1.56	0.34
2d	Treatment	1.62	0.78	1.12	8.28	0.82	2.24	0.00
	Control	1.44	0.67	0.92	9.02	0.65	1.49	0.42
4d	Treatment	1.50	0.72	0.99	8.93	0.86	1.77	0.49
	Control	1.42	0.66	0.96	8.70	0.61	1.28	0.00
6d	Treatment	1.64	0.72	1.02	9.42	0.72	1.67	0.47
	Control	1.58	0.72	1.00	9.04	0.68	1.38	0.52
8d	Treatment	1.42	0.68	0.95	9.40	0.64	1.42	0.48
	Control	1.38	0.66	0.92	8.92	0.62	1.37	0.44
10d	Treatment	1.37	0.64	0.94	10.15	0.62	1.22	0.52
	Control	1.42	0.66	0.92	9.37	0.64	1.37	0.52

Table 2. The average date of 17 kinds of amino acids.

Days after treatment		Valine	Methionine	Isoleucine	Leucine	Tyrosine	Phenylalanine
1d	Treatment	1.14	0.46	0.69	1.26	0.12	0.92
	Control	1.38	0.38	0.66	1.25	0.12	0.91
2d	Treatment	1.30	0.38	0.71	1.45	0.08	0.96
	Control	1.14	0.38	0.62	1.23	0.10	0.79
4d	Treatment	1.05	0.48	0.84	1.38	0.34	0.77
	Control	1.15	0.28	0.60	1.19	0.00	0.84
6d	Treatment	1.04	0.36	0.68	1.33	0.17	0.81
	Control	1.06	0.42	0.68	1.32	0.17	0.86
8d	Treatment	1.02	0.38	0.64	1.23	0.14	0.84
	Control	0.98	0.38	0.59	1.16	0.16	0.88
10d	Treatment	0.96	0.34	0.58	1.18	0.12	0.82
	Control	0.99	0.39	0.62	1.19	0.09	0.77

were calculated from three replicates (Tables 1-3).

RESULTS AND DISCUSSION

Effects of fungal elicitor on cell growth and triterpenoid production

Time courses of cell growth and triterpenoid accumulation of *B. platyphylla* Suk. cell cultures treated with 40 μg·L^{-1} fungal elicitor are shown in Figure 1. Compared with the control, the growth of cell cultures treated with the fungal elicitor was inhibited and significant inhibition ($P > 0.05$) was observed after a two-day elicitation. However, triterpenoid production in the treated cell cultures increased, reaching a maximum after 1-day elicitation, and then slowly declined thereafter. The maximum triterpenoid content and production in the treated cell cultures were 21.98 mg·g^{-1} and 123.82 mg·L^{-1} DW, respectively, which is two fold higher than that of the control without elicitation. After 1-day elicitation, the content and production of triterpenoids in the cell cultures treated with fungal elicitor decreased with increasing incubation time. These results could be attributed to

Table 3. The average date of 17 kinds of amino acids.

Days after treatment		Lysine	Histidine	Arginine	Proline	The content of total amino acid
1d	Treatment	1.01	0.50	0.92	0.84	22.51
	Control	1.01	0.56	0.95	0.73	22.89
2d	Treatment	0.54	1.66	0.84	1.25	24.03
	Control	0.49	1.70	0.77	1.02	22.85
4d	Treatment	0.45	1.64	0.86	1.06	24.13
	Control	0.62	1.52	0.64	0.96	21.43
6d	Treatment	0.48	1.64	0.81	1.14	24.12
	Control	0.46	1.61	0.78	1.12	23.40
8d	Treatment	0.49	1.66	0.78	1.05	23.22
	Control	0.48	1.62	0.86	0.99	22.41
10d	Treatment	0.46	1.58	0.70	1.00	23.20
	Control	0.46	1.66	0.70	1.06	22.83

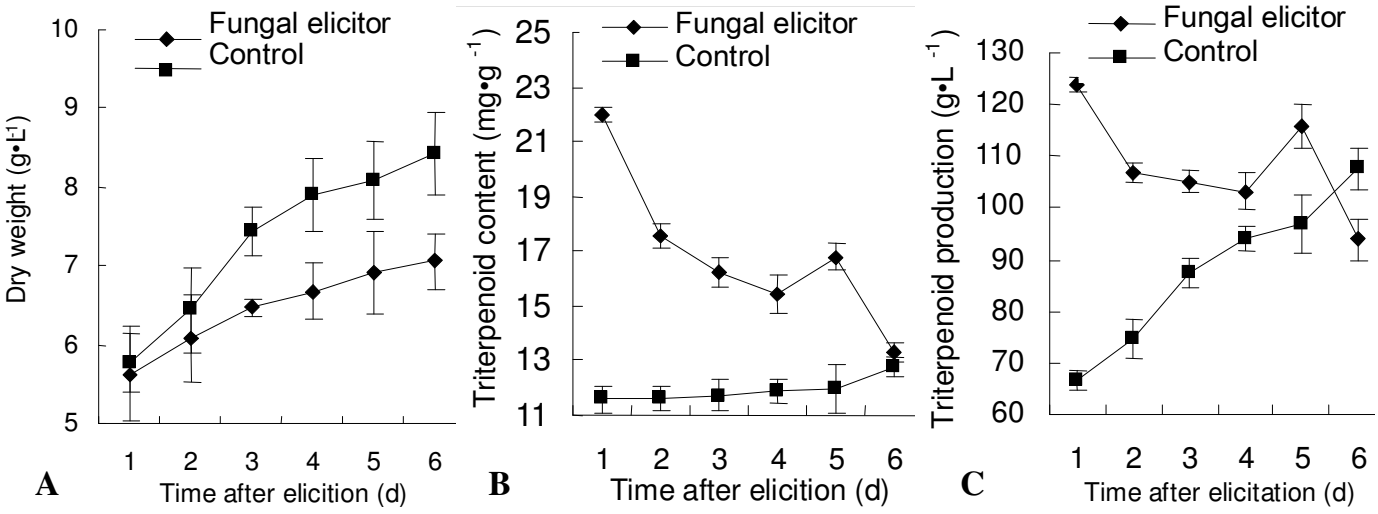


Figure 1. Time course of growth and triterpenoid accumulation of *Betula platyphylla* Suk. cell cultures after elicitation. Eight-day-old cells treated with 40 μg·L^{-1} elicitor were harvested after various periods; dry weight and triterpenoid production were then determined. The control received distilled water only. Data are mean ± SE of n = 3.

feedback inhibition of the triterpenoid synthetic enzymes caused by the great quantity of triterpenoids accumulated in cell cultures or to the competition for receptor sites between metabolites in the treated cell cultures, as well as the decrease of elicited stimulation or other mechanisms that are still unclear.

Effects of fungal elicitor on the soluble sugar content

The changes in soluble sugar content in *B. platyphylla* Suk. cell cultures induced with fungal elicitor are shown in Figure 2. The content of fructose, glucose, and total soluble sugar in the treated cell cultures increased and

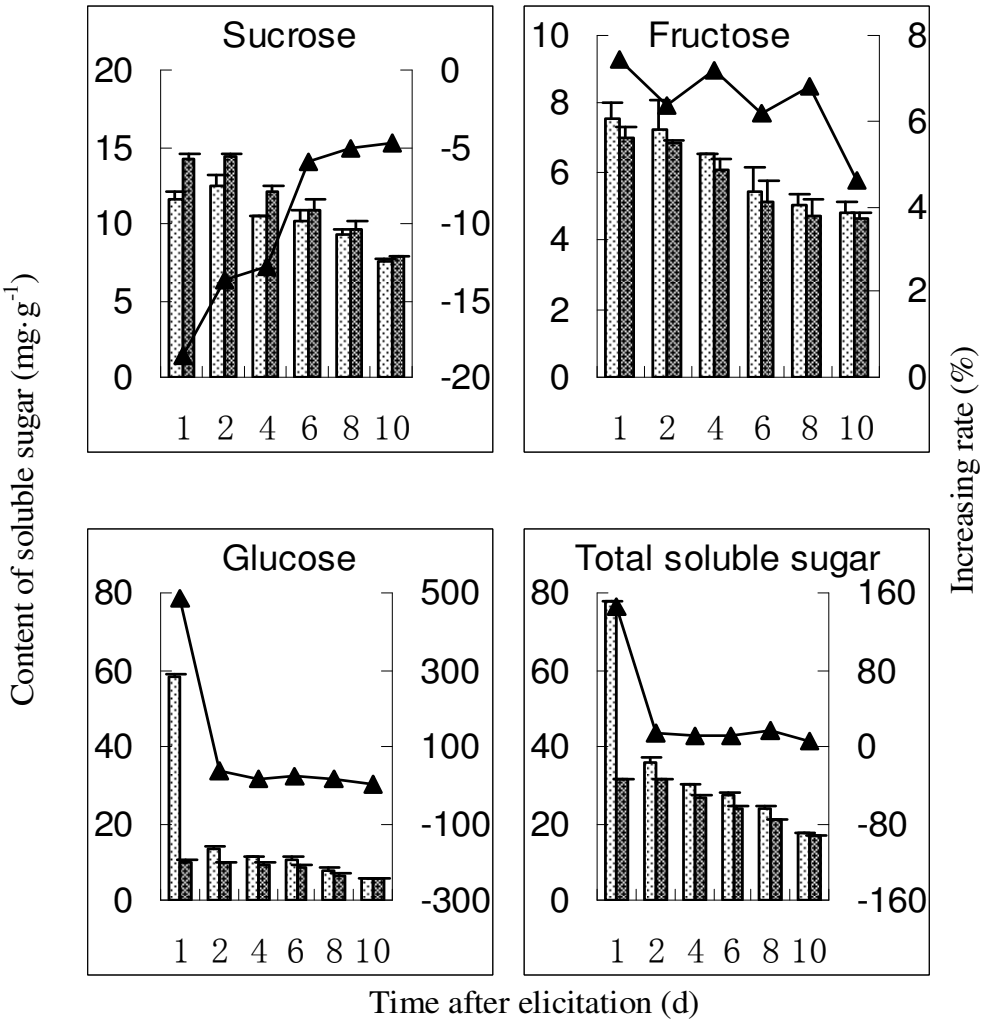


Figure 2. Changes in sucrose, fructose, glucose, and total soluble sugar content in the control (light color bar) and the fungal treatment cells (brunet bar) of *Betula platyphylla* Suk. The increasing rate (%) = (the value of treated cells-the value of control)/ the value of control × 100. Up to 40 μg·L^{-1} fungal elicitor was added to the medium on the 8th day. Data are mean ± SE of n = 3.

reached a maximum after 1-day elicitation, the rates of increase were 7.41, 484.54 and 146.93%, respectively. However, sucrose content in the treated cell cultures decreased and reached a minimum after 1-day elicitation, with an 18.62% rate of decrease. A similar phenomenon was also observed in suspension cultures of *Medicago truncatula*; MeJa elicitation increased total soluble sugar and glucose content, but showed decreased sucrose content in triterpene-saponin biosynthesis (Broeckling et al., 2005). In addition, the correlation analysis showed that the fungal elicitor significantly enhance the relationship of soluble sugars and triterpenoids (from -0.91* in the control to 0.95** in the treated cells), and had no significant effect on the individual content of soluble sugars and triterpenoids. This suggests that increased soluble sugar content may be pivotal in the ability of elicitors to stimulate secondary metabolite biosynthesis. Production of secondary metabolites by plant cells was affected by initial sugar concentration in a number of plant cell cultures, for example, in accumulation of alkaloid by *Cuthurunthus ruseus* (Merillon et al., 1984) and *Holurrhenu untidysenterica* cells (Panda et al., 1992), taxane production in suspension cultures of *Taxus chinensis* (Dong and Zhong, 2001), phenylpropanoid biosynthesis in grapevine cell cultures (Ferri et al., 2011). Unfortunately, the relationship between soluble carbohydrates and secondary metabolite biosynthesis is still unclear, especially under the elicitation treatment.

A possible explanation is that sugars are not only energy sources and structural components, but are also physiological signals regulating the expression of a variety of genes involved both in primary and secondary metabolism. It is known that sucrose and other sugars modulate the expression of many genes implicated in

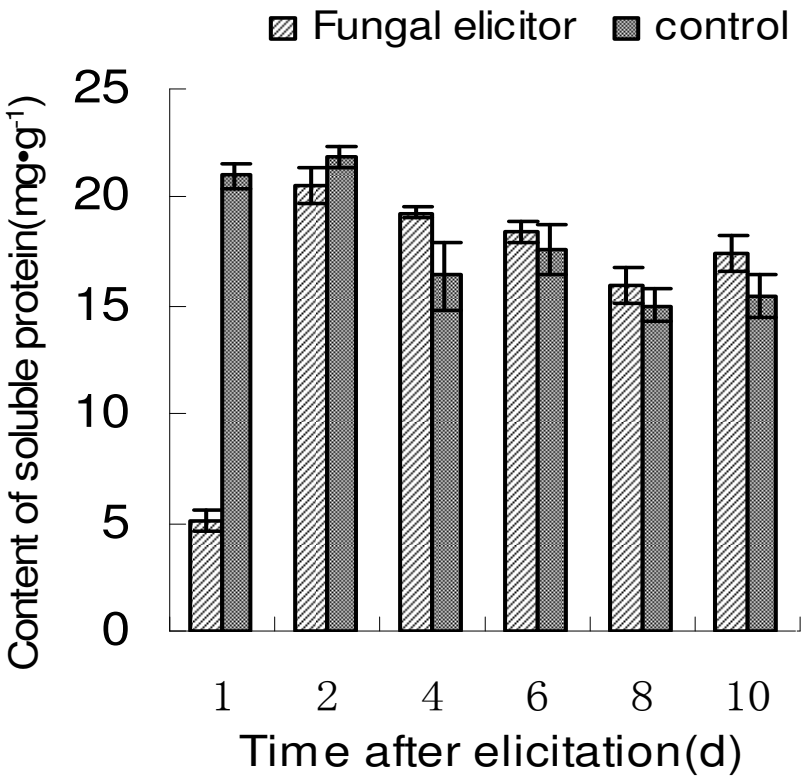


Figure 3. Changes in soluble protein in the control and the fungal treatment cells of *Betula platyphylla* Suk. Up to 40 μg·L^{-1} fungal elicitor was added to the medium on the 8th day. Data are mean ± SE of n = 3.

photosynthesis, respiration, nitrogen metabolism and defense processes (Jang et al., 1997). The sugar effect on plant cells seems to be due to the coupling of two mechanisms: osmotic stress and disturbed cellular metabolism (Do and Cormier, 1990). In addition, many sugar responses may be regulated by alterations in sugar flux (Koch, 2004; Smeekens et al., 2010) or in the C:N ratios (Coruzzi and Zhou, 2001; Mao et al., 2005) rather than by absolute sugar or sugar-metabolite levels. Further studies will help elucidate these questions.

Effect of fungal elicitor on soluble protein content

Soluble protein content in the treated cell cultures decreased, reaching a minimum after 1-day elicitation, after which it slowly increased (Figure 3). The minimum soluble protein content in the treated cell cultures was 5.15 mg·g^{-1} DW, which was 307.79% lower than that of the control without elicitation. This result is consistent with carbon-nutrient balance hypothesis (Coley et al., 1985; Van Dam et al., 1996) that secondary metabolism is directed towards carbon-rich metabolites in nitrogen-limited plants, and nitrogen-rich metabolites in carbon-limited plants. Low nitrogen typically leads to the accumulation of secondary metabolites, including phenylpropanoids and flavonoids (Van Der Werf et al., 1993; Hakulinen 1998; Cipollini et al., 2002; Lou and Baldwin, 2004). It is not known whether this is triggered by changes in nitrogen metabolism, accompanying changes in carbohydrates, or pleiotropic changes resulting from slow growth or dislocation of cellular functions (Fritz et al., 2006).

Effect of fungal elicitor on amino acid content

A total of 17 free amino acids were detected in the control and the treated *B. platyphylla* Suk. cells (Figure 4), four from the aspartate family (Asp, Ile, Lys and Thr), three from the glutamate family (Arg, Glu and Pro), two from the serine family (Gly and Ser), two from the aromatic family (Phe and Tyr), three from pyruvate family (Ala, Val, and Leu), and three other amino acids (Cys2, Met and His). Fungal elicitation increased the total amino acid content, especially in the glutamate, serine, and aromatic families, but it nearly had no effect on His, Cys2 and Lys. Among the 17 amino acids, Glu was the dominant in the control and the treated cells–it represented about 30% of the total free amino acids. Glu content ranged from 8.70% DW to 9.37% DW in the control and from 8.28 to 10.15% DW in the fungal-elicited cells. Fungal elicitation

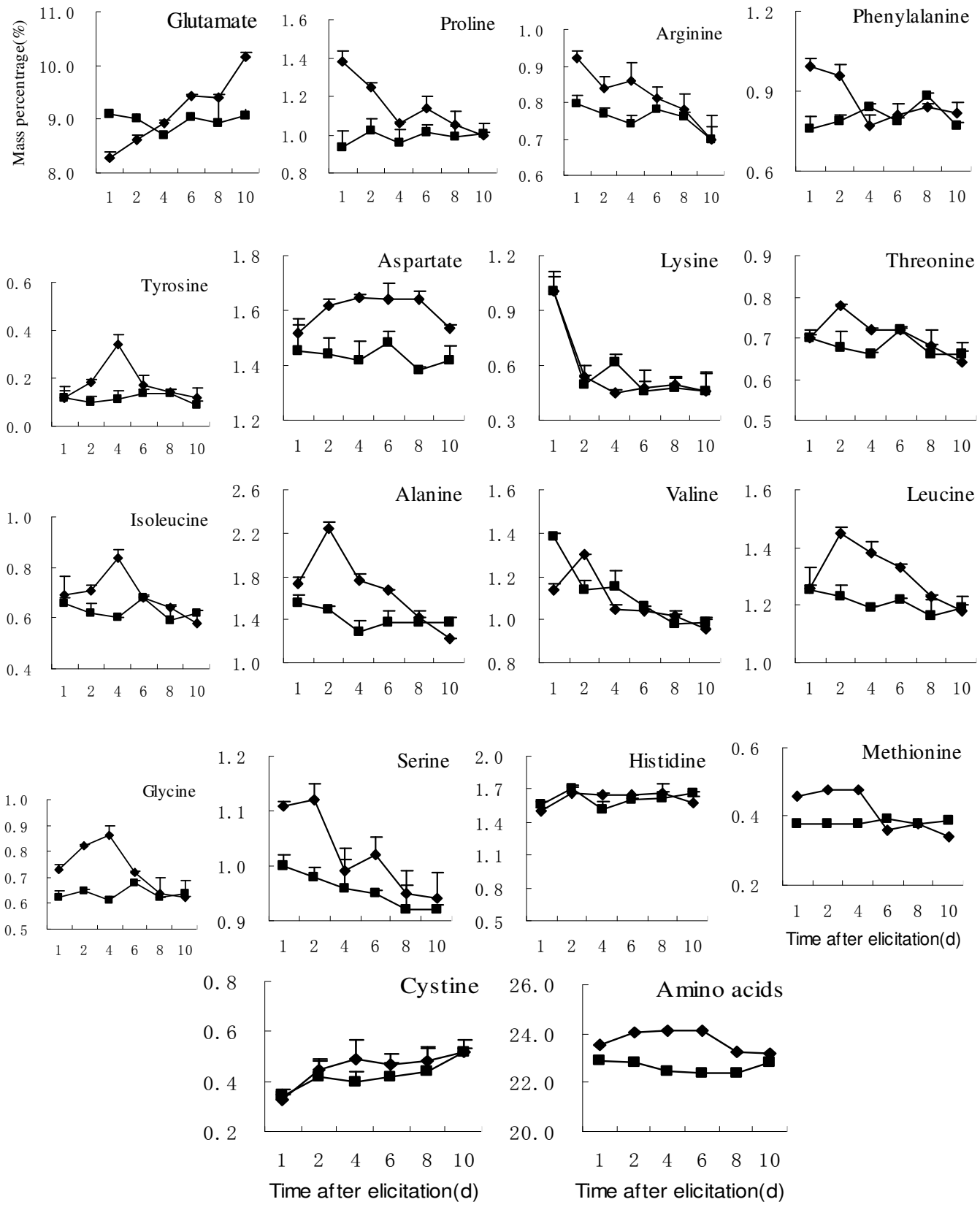


Figure 4. Changes in amino acid content in the control (squares) and fungal treatment cells (diamonds) of *Betula platyphylla* Suk. Up to 40 μg·L^{-1} fungal elicitor was added to the medium on the 8th day. Data are mean ± SE of n = 3.

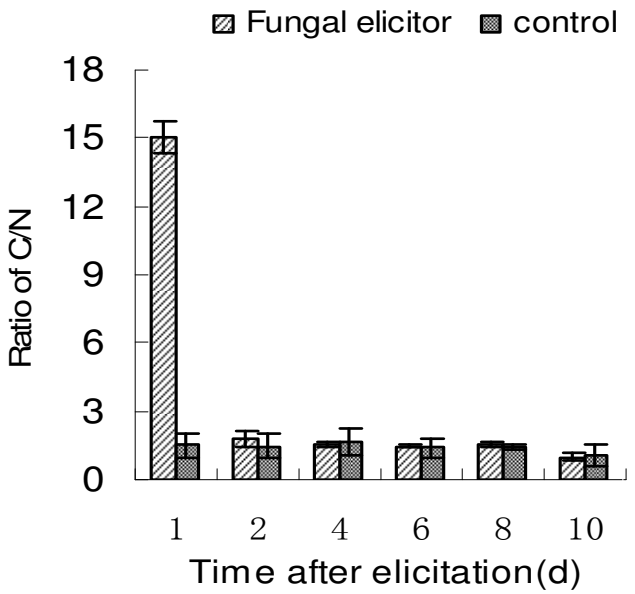


Figure 5. Changes in the C/N ratio in the control and the fungal treatment cells of *Betula platyphylla* Suk. Up to 40 μg·L^{-1} fungal elicitor was added to the medium on the 8th day. Data are mean ± SE of n = 3. C/N ratio is ratio of the concentration of soluble sugars to soluble proteins.

increased the total amino acid content, especially in the glutamate, serine and aromatic families. Further statistical analysis revealed that there was a significant negative correlation between Glu and triterpenoid content (from 0.46 in the control to -0.92** in treated cells), and no significant positive correlation occurs between other amino acids and triterpenoid content. Glu is a precursor for the synthesis of polyamine, and exogenous polyamine can stimulate the secondary metabolites production (Bais et al., 2004; Suresh et al., 2004; Yang et al., 2010). Thus, Glu may be pivotal in the ability of elicitors to stimulate triterpenoids synthesis. Future work is in progress to explore the effect of exogenous polyamine on the synthesis of triterpenoids.

Effect of fungal elicitor on C/N ratio

Fungal treatment resulted in a considerable increase in C/N ratio. The ratio reached its maximum, and then quickly decreased thereafter (Figure 5). The maximum C/N ratio in the treated cell cultures was 15.07, which is 906.96% higher than that of the control without elicitation (1.50). This suggests that a high C/N ratio may be an important factor in the ability of elicitors to stimulate secondary metabolite biosynthesis. The relationship of C/N ratio and secondary metabolism biosynthesis is an old question. To improve secondary metabolite production in non-treated plant cell cultures, optimization of carbon and nitrogen sources, as well as the carbon/nitrogen ratio is necessary (Mao et al., 2005; Liu and Wu, 2007). However, the function of C/N ratio on secondary metabolite production is still unclear. Many previous investigations mainly focused on C or N alone, and re-examination of those previously reported sugar or nitrogen responsive results in terms of C/N balance may be necessary to dissect the C/N signaling function. In addition, key components involved in C-N interactions in bacterial, yeast, and animal systems are clear; whether they are functionally conserved in plants is unclear (Zheng, 2009). Rapid advances in study methods will provide an important step towards the construction of complex yet elegant C/N balanced signaling networks in plants.

In conclusion, this study shows that the fungal elicitation technique is a useful strategy for stimulating triterpenoid production in *B. platyphylla* Suk. cell cultures. Changes of C and N status may be one of the key response factors in the ability of fungal elicitors to stimulate triterpenoid production. These hypotheses will be explained through integrated transcriptomic, proteomic and metabolomic studies.

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