DOI: 10.32615/bp.2019.053 BIOLOGIA PLANTARUM 63: 411-417, 2019

Growth, secondary metabolism, and related gene expression in response to interactions of nitrogen and sulfur in *Isatis indigotica*

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Abstract

Nitrogen and sulfur are major elements influencing plant growth and production of secondary metabolites. They interact to each other, but little is known about it in *Isatis indigotica* Fort. plants. In this study, 15 different treatments representing all possible combinations of 3 N treatments (N1, N2, and N3, corresponding to 5, 15, and 25 mM N, respectively) and five S treatments (S0, S1, S2, S3, and S4, corresponding to 0.00, 1.25, 2.50, 5.00 and 7.50 mM S, respectively) were used, and plant growth, indigo and indirubin yields, and expressions of genes encoding enzymes involved in N and S metabolisms were measured. The results show that the highest dry biomass was observed in N2S2 treatment. Moreover, net photosynthetic rate in the N2S2 treatment was significantly higher than under other treatments (except for N3S2 treatment). A low nitrogen concentration (5 mM) was beneficial to the accumulation of alkaloids, and the N1S1 and N2S2 treatments resulted in the highest yields of indigo and indirubin, respectively. Additionally, the yields of indigo and indirubin were positively correlated with the expression of *APS reductase* and *glutamine synthetase* genes, respectively.

Additional key words: indigo, indirubin, photosynthetic rate, RT-qPCR, stomatal conductance, transpiration rate.

Introduction

Mineral nutrition affects metabolic processes in plants and thus also the useful products of secondary metabolism (Herms *et al*. 1992). Nitrogen and sulfur are two essential macronutrients and function in numerous processes (Kopriva *et al*. 2015). It has been suggested that the accumulation of alkaloids in plant tissues is closely correlated with N concentration in the soil. For example, the vinblastine and vincristine content of *Catharanthus roseus* are highest at a moderate nitrogen concentration (Singh *et al*. 2015). In *Sencio jacobaea*, the content of total viroxane alkaloids decrease significantly as N concentration increases (Hol *et al*. 2003). Previous studies demonstrated that a low concentration of inorganic N in the soil reduces S uptake and assimilation (Davidian *et al*. 2010). Also, it has been reported that S deprivation results in a disruption of N metabolism by a reduction of nitrate reductase activity (De Bona *et al*. 2011, Sorin *et al*. 2015) or by a limitation of protein synthesis (Hesse *et al*. 2004). Thus, optimal nutrition can affect primary metabolism and subsequently regulates secondary metabolism including alkaloids.

Medicinal plants play an important role in the prevention and treatment of human diseases. Thus, the cultivation of medicinal plants does not focus only on yields but also on the content of secondary metabolites. *Isatis indigotica* Fort. is a biennial herbaceous plant of the *Cruciferae* family, which is widely distributed and cultivated in China. Its dry leaves are used in a traditional Chinese medicine. The pharmacological activity of *I. indigotica* is due to its alkaloids, organic acids, glycosides, sterols, *etc* (Zhou and Zhang 2013). The most important components are indirubin and indigo. Besides the work on their isolation and therapeutic potential, research is needed to enhance the production of

Submitted 24 July 2018, *last revision* 11 December 2018, *accepted* 13 December 2018.
Abbreviations: APR - APS reductase; APS - adenosine phosphosulfate; ATPS - ATP sulfurylase; c_i - intercellular CO_2 concentration; E - transpiration rate; GDH - glutamate dehydrogenase; GOGAT - glutamate synthase; g_s - stomatal conductance; GS - glutamine synthetase; NR - nitrate reductase; OAS-TL - cysteine synthase; P_N - net photosynthetic rate.
Acknowledgments: This work was supported by the National Natural Science Foundation of China (Grant no. 31171486).
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of medicinally important metabolites. In our previous report, we have established the role of N forms and their content in biomass yield, and indigo and indirubin accumulations (Xiao *et al*. 2013). Previous studies have paid little attention to the interactions of N and S on the growth and metabolism of *I. indigotica.* Furthermore, they have not clarified how the expressions of genes involved in the metabolism of N and S respond to different applications of N and S. The present study aims to assess the effects of different ratios of N and S on growth, indigo and indirubin yields, and gene expressions of enzymes involved in the metabolism of N and S in *I. indigotica* seedlings. Data generated by this study were expected to be of a great value for understanding the effects of N and S on indigo and indirubin yields in *I. indigotica*.

Materials and methods

Plant growth and treatments: A two-factor completely randomized experiment with four replicates was conducted in the Nanjing Agricultural University in China, between April and July 2017. *Isatis indigotica* Fort. plant were cultivated under a natural irradiance at a maximum day temperature of 32 °C and a minimum night temperature of 15 °C, and a 60 - 80 % air humidity. Seeds of *I. indigotica* were sown in pots filled with *Vermiculite* and *Perlite* (2:1, v/v), and seedlings at the six leaves stage were transplanted into plastic pots filled with 10 kg of quartz sand (10 seedlings/pot). The quartz sand had been soaked with 1 % (m/v) HCl for 24 h, and then rinsed before use.

The seedlings were subjected to 15 different treatments representing all possible combinations of 3 N concentrations (N1, N2, and N3 corresponding to 5, 15, and 25 mM N,respectively) and 5 S concentrations (S0, S1, S2, S3, and S4, corresponding to 0, 1.25, 2.50, 5.00, and 7.50 mM S, respectively) (Table 1 Suppl.). The nitrogen and sulfur of nutrient solution were provided by ammonium nitrate (NH_4NO_3) and the sulfate ion (SO_4^{2-}), and the nitrogen and sulfur concentrations were controlled by changing the amount of NH_4NO_3, $MgSO_4 \cdot 7\,H_2O$, K_2SO_4, and $CaSO_4$. All reagents were of analytical grade. Prior to applying the fertilizers, the plants were maintained for five days in double distilled water and then regularly supplied with a reformed Hoagland solution (Hoagland and Arnon 1950).

After 45 d, 10 seedlings selected from each treatment were randomly collected. Three samples were oven-dried firstly at 105 °C for 15 min and then at 65 °C until a constant mass was achieved. Fresh leaves were also stored at -80 °C.

Determination of photosynthetic parameters: Net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (c_i), and transpiration rate (E) of the third leaves from inside out were measured under an irradiance of 1 000 μmol m^{-2} s^{-1} with a *LI-6400* portable photosynthesis system (*LI-COR*, Lincoln, NE, USA). Five leaves were measured for each treatment.

Extraction and quantification of indigo and indirubin: Indigo and indirubin were extracted by the Soxhlet method. A leaf powder (0.1 g) packed in fat-free filter paper was soaked chloroform for 15 h, and then put into tube extractors with chloroform and heated until the extraction solution became colourless. After the chloroform volatilized, the samples were dissolved in methanol and transferred to 10-cm^3 flasks, diluted with 10 cm^3 of methanol, and mixed. The samples were then filtered using 0.45 μm microfiltration membranes.

Ultra performance liquid chromatography was conducted using a method specifically developed for Chinese pharmacopeia (Chinese Pharmacopoeia Commission 2015), and an *Agilent ZORBAX-Eclipse-Plus* C_{18} column (2.1 mm × 50 mm, 1.8 μm film thickness) was used. Column temperature was 30° C. The mobile phase was consisted of methanol and water (72:28, v/v) and set at a flow rate of 0.3 $cm^3 \cdot min^{-1}$. Samples of 2 mm^3 were automatically injected and analyzed using UV radiation at 289 nm. Each compound was quantified using a specific external standard.

Gene selection, primer design, and RT-qPCR: Seven target genes and one reference gene from the *I. indigotica* transcriptome data were identified. Primers were designed based on their sequences using the *Primer Premier 5.0* software with following criteria: a GC content of 45 - 65 %, an optimal melting temperature of 58 - 61 °C, a primer length of 19 - 26 bp, and an amplicon length of 80 - 200 bp (Table 2 Suppl.). Primer specificity was judged by melting-curve analysis and 2 % (m/v) agarose gel electrophoresis of the amplification products (Fig. 1 Suppl.).

Total RNA from various tissue samples was isolated using an *RNA simple total RNA* kit (*Tiangen*, Nanjing, China). The RNA concentration of each sample was determined using a *NanoDrop-2000* spectrophotometer (*Thermo Fisher Scientific*, Wilmington, USA). Samples with a 260/280 nm ratio between 1.9 and 2.1, and a 260/230 nm ratio ≥ 2.0 were chosen to determine the quality and purity of the RNA preparations. The integrity of the purified RNA was checked by 1.0 % agarose gel electrophoresis. Subsequently, the first-strand cDNA was synthesized from RNA (300 ng mm^{-3}) in 20 mm^3 of a reaction mixture using a *PremeScript*™ RT reagent kit with a gDNA eraser (*TaKaRa*, Dalian, China) following the manufacturer's protocol.

Three biological replicates from each treatment were subjected to the reverse transcription quantitative PCR

assay using the *SYBR Green* methodology and an *ABI 7500* real-time PCR system (*Applied Biosystems*, Foster City, USA) with *Actin* as a reference gene. A PCR reaction was prepared in a 20 mm^3 volume containing: 2 mm^3 of a 5-fold diluted synthesized cDNA, 10 mm^3 of a real-time PCR *Master Mix*, 2 mm^3 of each primer, 0.4 mm^3 of ROX1 (50×), and 5.6 mm^3 of double distilled H_2O. A reaction comprised an initial step at 95 °C for 10 min followed by 40 denaturation cycles at 95 °C for 5 s and primer annealing at 60 °C for 30 s. Next, melting curves ranging from 60 to 95 °C were evaluated in each reaction to check the specificity of the amplification products. Relative fold expression changes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis: Data were statistically analyzed using the *SPSS* software (*v. 19.0*; *IBM*, USA), and tested for homogeneity of variance by means of least significant difference multiple range tests at the level of 0.05. Graphs were drawn with the *Origin* (*v. 9.0*; *Origin Lab*, Massachusetts, USA).

Results

Plants were irrigated with a nutrient solution with different N and S ratios. For the N2S2 treatment, total dry mass per plant was significantly higher than in other treatments, about 2-times higher (Fig. 1). It shows that a proper N and S application can indeed optimize plant growth. The effect of N or S applications on the total dry mass suggests that moderate concentrations were most appropriate, and the N2 and S2 treatments generated the highest plant growth.

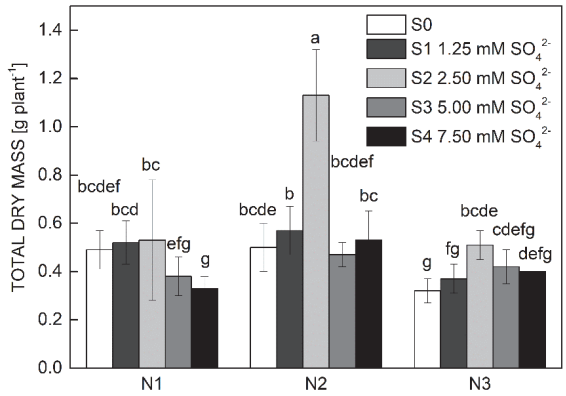

Fig. 1. Total dry mass of *Isatis indigotica* leaves exposed to different concentrations of nitrogen (N1, N2, and N3: 5, 15, and 25 mM NH_4NO_3, respectively) and different concentrations of sulfur (S0: 0.00; S1: 1.25 mM $MgSO_4$; S2: 2.50 mM $MgSO_4$; S3: 2.50 mM $MgSO_4$ and 2.50 mM K_2SO_4; S4: 2.50 mM $MgSO_4$, 2.50 mM K_2SO_4, and 2.50 mM $CaSO_4$). Means ± SDs, n = 10, values followed by different letters are significantly different ($P \leq 0.05$).

Net photosynthetic rate depended on S concentration, P_N firstly increased and then decreased when the S concentration increased (Table 1). However, the effect of N concentration on P_N was not significant. Overall, P_N in the N2S2 and N3S2 treatments was higher than under other treatments, which indicates that suitable N and S ratios could effectively improve P_N promoting dry mass accumulation in *I. indigotica* seedlings. On the contrary, intercellular CO2 concentration was obviously lower under the N3S2 treatment than in other treatments (except for the N3S0). There were no significant differences among most treatments. Stomatal conductance and transpiration rate firstly increased and then decreased as the S concentration increased (Table 1).

The highest yields of indigo and indirubin were 7.38 $\mu g \cdot g^{-1}$(plant d.m.) and 2.58 $\mu g\ g^{-1}$(plant d.m.) in the N1S1 and N2S2 treatments, respectively. However, under a higher N concentration, a significant decrease in the yields of indigo and indirubin were observed. Also, a high concentration of S caused a reduction in the yields of indigo and indirubin, and the moderate S application increased them (Table 2).

When analyzing the transcription of the different genes related to N metabolism enzymes, we observed that the N3 significantly increased expression of the *nitrate reductase* (*NR*) gene: its relative expression increased by about 3.2-, 11.0-, 9.3-, 11.3-, and 15.2-fold under S0, S1, S2, S3, and S4 treatment groups, respectively, in comparison to N1 (Fig. 2*A*). The *glutamine synthetase* (*GS*) and *glutamate synthase* (*GOGAT*) genes were strongly down-regulated under the N3 concentration (Fig. 2*B*,*D*). Meanwhile, the positive effect of the moderate N concentration (N2) on *glutamate dehydrogenase* (*GDH*) gene expression was found (Fig. 2*C*). Comparing gene expressions with increasing S concentration, it was found that *NR*, *GS*, *GDH*, and *GOGAT* genes mostly first increased and then decreased. For example, at N2, the largest expression of the *GS* gene was observed in combination with S2, which was 4.7-, 1.5-, 4.1-, and 92.0-fold higher in comparison to S0, S1, S3, and S4, respectively (Fig. 2*B*). Finally, no significant difference was observed in expression of *GS* and *GOGAT* genes under the N3 treatment (Fig. 2*BD*).

The expression of genes related to S metabolism enzymes did not follow a fixed pattern under different treatments. The relative expression of the *adenosine phosphosulfate reductase* (*APR*) gene was significantly up-regulated under N1, which was 7.7-, 18.2-, 8.2-, 6.1- and 6.8-fold, respectively, higher than under N3 (Fig. 3*B*). But, no clear change of *ATP sulfurylase* (*ATPS*) and cysteine synthase (*OAS-TL*) gene expressions were found under different N applications. The *ATPS* and *APR* gene relative expressions were significantly up-regulated under

Table 1. Effect of different nitrogen and sulfur treatments on net photosynthetic rate (P_N), stomatal conductance (g_s), intecellular CO_2 concentration (c_i), and transpiration rate (E) of *Isatis indigotica*. Means ± SDs, $n = 5$. Different letters indicate significant differences between treatments using the Dunnett test at $P \leq 0.05$. For different treatments, see Fig. 1.

Treatments	P_N [μmol·m^{-2}·s^{-1}]	g_s [mol·m^{-2}·s^{-1}]	c_i [μmol·mol^{-1}]	E [mmol·m^{-2}·s^{-1}]
N1S0	11.22 ± 0.77fg	0.30 ± 0.05bc	313.71 ± 7.86a	5.33 ± 0.55abc
N1S1	13.37 ± 0.29abc	0.35 ± 0.04bc	310.25 ± 6.78a	5.59 ± 0.34abc
N1S2	14.00 ± 0.89ab	0.43 ± 0.10ab	314.69 ± 9.68a	6.70 ± 0.83ab
N1S3	13.23 ± 0.93abcd	0.42 ± 0.05ab	317.88 ± 1.26a	6.74 ± 0.37ab
N1S4	10.72 ± 0.69g	0.28 ± 0.11bc	302.93 ± 23.32ab	5.27 ± 1.20abc
N2S0	11.75 ± 0.01defg	0.54 ± 0.00a	324.99 ± 0.06a	6.28 ± 0.00ab
N2S1	11.95 ± 0.00cdefg	0.19 ± 0.00c	263.30 ± 0.06cd	4.00 ± 0.00c
N2S2	14.29 ± 0.41a	0.44 ± 0.15ab	307.44 ± 21.06a	6.88 ± 1.27a
N2S3	11.64 ± 2.00efg	0.34 ± 0.24bc	295.61 ± 39.49ab	5.48 ± 2.44abc
N2S4	11.45 ± 1.28efg	0.39 ± 0.17ab	316.36 ± 13.02a	6.31 ± 1.59ab
N3S0	12.62 ± 0.31bcdef	0.18 ± 0.04c	244.12 ± 21.76d	4.06 ± 0.74c
N3S1	12.89 ± 0.45abcde	0.55 ± 0.10a	321.45 ± 6.85a	6.43 ± 0.49ab
N3S2	14.31 ± 1.16a	0.29 ± 0.03bc	277.40 ± 10.81bc	5.86 ± 0.60ab
N3S3	11.65 ± 0.40efg	0.35 ± 0.06bc	306.51 ± 11.40a	4.99 ± 0.39bc
N3S4	10.86 ± 0.58g	0.34 ± 0.04bc	309.12 ± 7.63a	5.00 ± 0.40bc

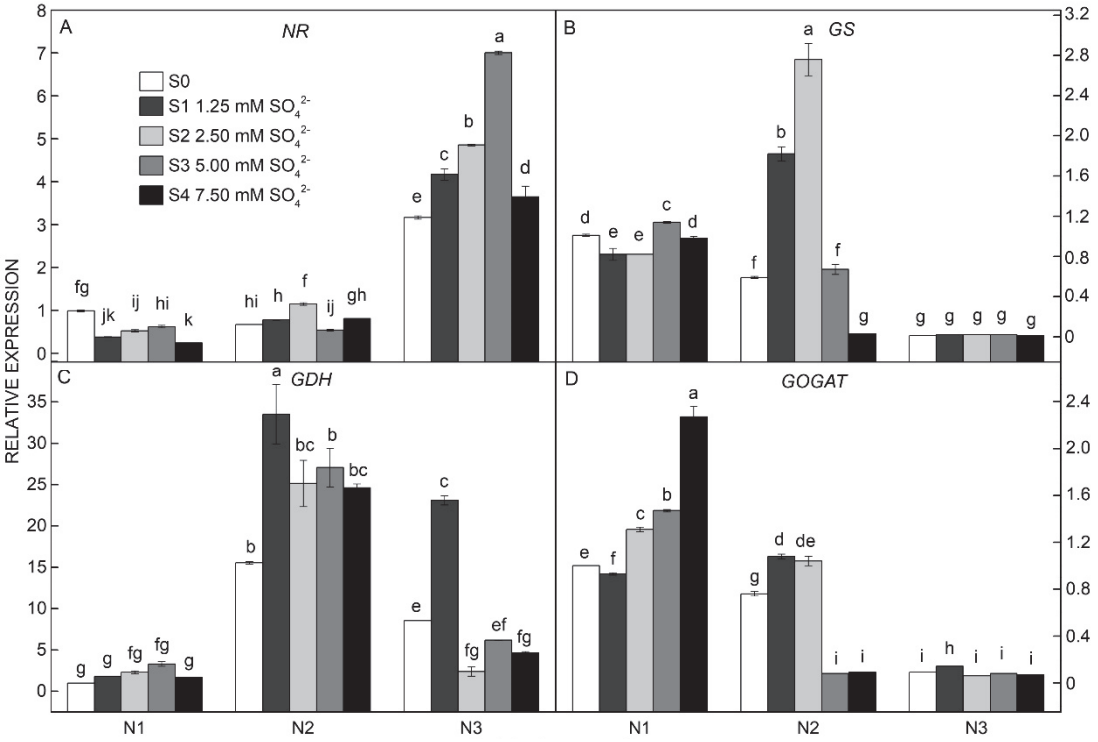

Fig. 2. Relative expressions of genes encoding nitrogen metabolism enzymes of *Isatis indigotica* under different concentrations of nitrogen and sulfur. Means ± SDs, $n = 3$, different letters indicate significant differences ($P \leq 0.05$). For different treatments, see Fig. 1. *NR - nitrate reductase*, *GS - glutamine synthetase*, *GDH - glutamate dehydrogenase*, *GOGAT - glutamate synthase*.

S deficiency (Fig. 3*ABC*).

Correlation analysis shows that the expression of the *GOGAT* gene was positively correlated with the expressions of the *GS* gene, and *APR* gene (Table 3), whereas the expression of the *NR* gene was negatively correlated with the expressions of the *GS*, *GOGAT*, and *APR* genes. Additionally, the expression of the *GDH* gene was negatively correlated with the expression of the *APR* gene. Interestingly, the indigo and indirubin yields were positively correlated with the expressions of *APR* and *GS* genes, respectively. Moreover, under different N and S concentrations, the indigo and indirubin yields showed a

significant mutual correlation. It was suggested that the accumulation of products of secondary metabolism might be correlated with the primary metabolic enzyme expressions in *I. indigotica*.

Principle component analysis aimed to establish relationships between different ratios of N and S based on the biochemical parameters and the expressions of genes related to the metabolism of N and S (Fig. 4.). The analysis showed that *1*) there were differences between treatments as indicated by the degrees of separation, *2*) there were clear interactions between N and S as indicated by separation between treatments at a given level of N or S, and *3*) the N2S2 treatment clearly stood out from other treatments.

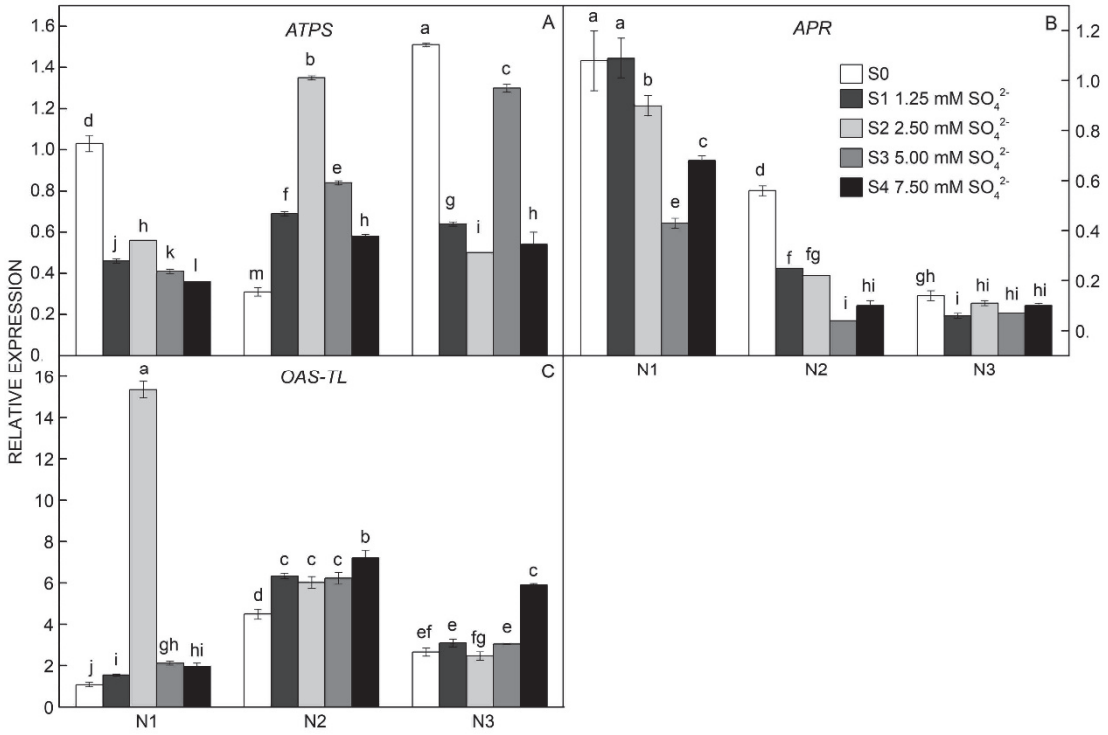

Fig. 3. Relative expressions of genes encoding sulfur metabolism enzymes in *Isatis indigotica* under different concentrations of nitrogen and sulfur. Means ± SDs, $n = 3$, different letters indicate significant differences ($P \leq 0.05$). For different treatments, see Fig. 1. *ATPS - ATP sulfurylase*, *APR - adenosine phosphosulfate reductase*, *OAS-TL* - cysteine synthase.

Discussion

In this investigation, the accumulation of dry matter in *I. indigotica* was promoted under 15 mM N and 2.5 mM S, and no significant difference was found among other treatments (Fig. 1). The positive effects of suitable N and S ratios on growth and biomass accumulation was coincident with previous reports in other medicinal and crop plants (Salvagiotti *et al*. 2009, Tsujimoto *et al*. 2017, Raza *et al*. 2018). The reason might be an enhanced nitrogen use efficiency (Salvagiotti and Miralles 2008). In addition, the increase of biomass production required an increase in photosynthetic carbon fixation as in average 40 % saccharides form dry mass of wheat (Murchie *et al*. 2009, Parry *et al*. 2011). Here, we observed that P_N in the N2S2 treatment was significantly higher than in other treatments (except for the N3S2 treatment) (Table 1), which was correlated with the total dry mass. Thus, we assume that the accumulation of the total dry mass in *I. indigotica* could be enhanced by improving the efficiency of photosynthetic carbon fixation under an optimal N/S ratio.

Alkaloids are important N-containing secondary metabolites. At present, there are about 1 600 known alkaloids (Wink 2003) and indigo and indirubin belong to the indole alkaloid family. Therefore, the biosyntheses of indigo and indirubin are directly or indirectly related to the N and S content. The highest yields of indigo and indirubin were found under a moderate N concentration (Table 2). The higher N concentrations facilitated plant growth and N metabolism, but failed to increase the accumulation of secondary metabolites. Guan *et al*. (2018) also reported that low nitrogen applications promote indigo and indirubin content. Moreover, the accumulation of secondary metabolites is also influenced by transportation and distribution among cells or tissues, and also by their degradation (Zhao *et al*. 1999). These are complex processes that need to be further studied. Optimal N and S

ratios are therefore important for the cultivation of medicinal plants.

Nitrogen is a vital component of living matter (Rentsch *et al*. 2007) and S is considered as the fourth main plant nutrient after nitrogen, phosphorus, and potassium (Scherer 2001). It is well known that N and S are essential in primary and secondary metabolism (Aharoni and Galili 2011, Maeda and Dudarev 2012, Giordano and Raven 2014). As expected, the results obtained by correlation analysis show that the yields of indigo and indirubin were correlated with the expressions of *APR* and *GS* genes (Table 3). As a key enzyme in the S metabolism pathway, APR plays an important role in synthesis of amino acids or proteins (Leustek and Saito 1999, Leustek *et al*. 2000). Tryptophan is a precursor of indigo. Thus, APR may affect the synthesis of indigo. The synthesis pathway of indirubin needs some important precursors including indoxyl, indole, anthranilate, and chorismate (Marcinek *et al*. 2000). Anthranilate synthesis is closely related with ammonium (Mentzen *et al.* 2008, Gerhards *et al*. 2014, Xu *et al*. 2014) and GS is an N metabolism enzyme which also participates in the metabolism of ammonium (Miflin 1974, Fernández *et al*. 1998, Rosales *et al*. 2011). Thus, it may explain the relationship of indirubin yield with expression of the *GS* gene. The expression of the *NR* gene increased with increasing N concentration, whereas expressions of *GS*, *GOGAT*, and *APR* mostly decreased. Principle component analysis results indicate that N2S2 was the optimal nutrition of *I. indigotica*. Manipulating the ratio of N and S might be an effective way to achieve higher yields of indigo and indirubin.

Table 2. The efficient yields of alkaloids indigo and indirubin as influenced by interaction of nitrogen and sulfur. Means ± SDs, n = 3, different letters indicate significant difference between treatments using the Dunnett test at $P \leq 0.05$. For different treatments, see Fig. 1.

Treatments	Indigo[μg·g^{-1}(d.m.)]	Indriubin[μg·g^{-1}(d.m.)]
N1S0	3.45 ± 0.18d	1.18 ± 0.00d
N1S1	7.38 ± 0.11a	1.97 ± 0.06b
N1S2	6.83 ± 0.10b	1.84 ± 0.02c
N1S3	2.23 ± 0.11e	1.15 ± 0.02d
N1S4	1.20 ± 0.04g	0.36 ± 0.03ij
N2S0	0.60 ± 0.09j	0.54 ± 0.01h
N2S1	0.60 ± 0.04j	0.59 ± 0.00g
N2S2	4.93 ± 0.19c	2.58 ± 0.06a
N2S3	1.47 ± 0.15f	0.60 ± 0.00g
N2S4	0.61 ± 0.02j	0.55 ± 0.02h
N3S0	0.91 ± 0.01i	0.85 ± 0.01f
N3S1	1.11 ± 0.03gh	0.94 ± 0.01e
N3S2	1.43 ± 0.04f	0.83 ± .01f
N3S3	1.03 ± 0.01hi	0.39 ± 0.02i
N3S4	0.92 ± 0.00i	0.33 ± 0.00j

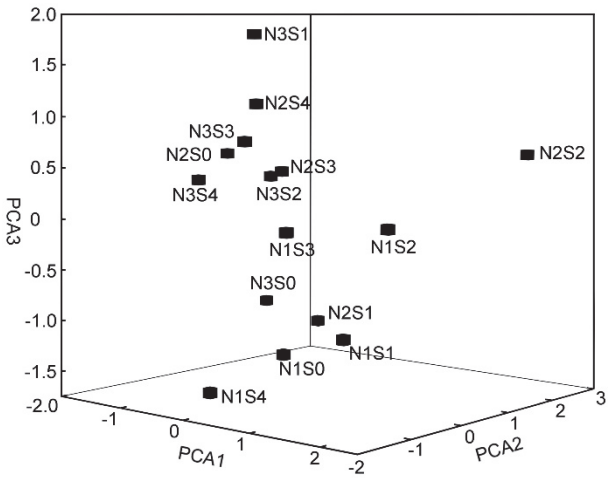

Fig. 4. Principle component analysis of biochemical parameters and gene expressions related to nitrogen and sulfur metabolism under various nitrogen and sulfur concentration in *Isatis indigotica*. For different treatments, see Fig. 1.

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