

The mechanisms underlying melatonin improved soybean seedling growth at different nitrogen levels

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ABSTRACT

To investigate the function of melatonin (MT) on nitrogen uptake and metabolism in soybean, six groups of treatments, with and without 100 μ M melatonin were conducted at low, normal, and high nitrogen levels (1.5, 7.5, and 15 mM, respectively). The related indexes of nitrogen metabolism and the antioxidant system of seedlings were measured and analysed. Results indicated that MT could enhance the level of nitrogen metabolism by upregulating the coding genes of enzymes related to nitrogen metabolism and increasing total nitrogen content, especially under low nitrogen levels. Under high nitrogen conditions, the addition of MT not only accelerated ammonium assimilation and utilisation by enhancing the activity of glutamine synthetase involved in ammonium assimilation, but also reduced the extent of membrane lipid peroxidation to alleviate the degree of damage by improving the activity of antioxidant enzymes. In addition, MT enhanced soybean growth with positive effects in morphological changes at different nitrogen levels, including significantly increased stem diameter, total leaf area, and root nodule number, and biomass accumulation. Finally, biomass accumulation increased under low, normal, and high nitrogen levels by 9.80%, 14.06%, and 11.44%, respectively. The results suggested that MT could enhance the soybean tolerance to low and excessive N treatments.

Keywords: crop physiology, legumes, melanin, nitrogen inappropriate, nitrogen metabolism, nitrogen nutrition, nitrogen regulation, soybean.

Introduction

Nitrogen is an essential nutrient element since its availability is closely related to crop growth, yield, and stress responses (Arun *et al.* 2016). As a nitrogen-loving crop, soybean is highly dependent on nitrogen nutrition (Sinclair and de Wit 1975; Wang *et al.* 2017). Although soybean has the unique ability of facilitating symbiotic nitrogen fixation with root nodules under low soil nitrogen conditions, it cannot always achieve the high nitrogen demand of soybean (Harper 1974; Ray *et al.* 2006; Cafaro La Menza *et al.* 2017), especially because the N fixation ability of root nodules is weak, and the source of nitrogen mainly dependent on exogenous application (Bergersen 1958). Under conditions of nitrogen deficiency, nitrogen metabolism in plants will be reduced, leading to the decline of other physiological metabolic processes such as photosynthesis and inhibiting overall growth and development (Takahashi *et al.* 2005). In addition, nitrogen restriction inhibits yield potential. According to the soybean yield potential, the model of nitrogen requirement to simulate total nitrogen supply has been used (Tamagno *et al.* 2017). The results showed that the yield of zero-N treatment was significantly lower than that of full-N treatment by 11% (Cafaro La Menza *et al.* 2017). However, if nitrogen fertiliser application is too great, the excess ammonium accumulation will cause toxicity, resulting in an interference in physiological and metabolic processes and yield reduction (Guan *et al.* 2016). A study on the effect of high nitrogen on wheat also demonstrated that the application of superabundant nitrogen fertiliser led to excessive accumulation of reactive oxygen species (ROS), aggravated the degree of membrane lipid peroxidation,

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significantly interfered with metabolic activities in the cell, and finally resulted in the reduction of wheat yield (Kong *et al.* 2017). Additionally, it may also increase the risk of large-scale plant lodging and is not conducive to the symbiotic nitrogen fixation function of soybean (Ciampitti and Salvagiotti 2018). Therefore, excessive supply of nitrogen fertiliser will reduce the utilisation rate of nitrogen fertiliser, pare off the yield, and cause environmental pollution (Xu *et al.* 2012; Yong *et al.* 2018). In addition, the high nitrogen concentration in the soil could be suitable for increasing the possibility of infection by pathogens such as soybean root rot (Liu *et al.* 2016a). Therefore, either a lack or an excess of nitrogen is not beneficial to the growth and development of crops.

To reduce the effect of nitrogen deficiency or excessive application on soybean during cultivation, it is necessary to find a method to solve this issue. Currently, many researchers are attempting to improve the biological yield of soybean under nitrogen stress through the cultivation of new varieties tolerant to nitrogen stress (Nawaz *et al.* 2018; Xie *et al.* 2019). Additionally, a large number of experiments have been conducted to enhance the utilisation of nitrogen fertiliser and increase yield by optimising nitrogen management (Du *et al.* 2020), using crop rotation and intercropping, or improving field management measures (Grassini *et al.* 2014; Gavili *et al.* 2019; Raza *et al.* 2019). In recent years, some researchers have begun to pay attention to the regulatory effects of plant growth regulators on crop growth and development under different nitrogen concentrations (Du *et al.* 2020). Melatonin, a newly discovered type of plant growth regulator with a similar effect to auxin (Josefa *et al.* 2004; Arnao and Hernández-Ruiz 2019), has been widely confirmed to promote root development (Liang *et al.* 2017; Chen *et al.* 2018), benefit plant growth (Byeon and Back 2014), delay leaf senescence (Park and Back 2012), and increase yield significantly (Liu *et al.* 2016b; Hu *et al.* 2018).

In addition, melatonin can function as an antioxidant capacity. In recent years, melatonin has been widely used as a protective agent against multiple stresses including unfavourable salt concentrations, ultraviolet radiation, toxic chemicals, extreme temperature, osmotic stress, pathogen infection, and nutritional defects (Zhang *et al.* 2017a; Fan *et al.* 2018; Bawa *et al.* 2020; Mohamed *et al.* 2020; Zhang *et al.* 2020). Under abiotic stress including drought, the alleviation effect of melatonin on plant growth may also be attributed to its regulation of carbon and nitrogen metabolism (Cao *et al.* 2019; Zou *et al.* 2020). Some studies have pointed out that melatonin could play a role in plants by enhancing cell division, promoting photosynthesis and carbohydrate synthesis, and other carbon metabolic pathways (Wei *et al.* 2015). Furthermore, a study on the application of melatonin under the condition of nitrogen deficiency confirmed that melatonin could increase biological yield by participating in the pathway of nitrogen uptake and assimilation of wheat (Qiao *et al.* 2019). However, under excessive nitrogen

supply conditions, the use of melatonin could also effectively inhibit the accumulation of inorganic nitrogen ions in plant leaves (Zhou *et al.* 2019). A study on the molecular regulation mechanism of exogenous hormone in *Malus* under nutritional stress has explored that melatonin could not only promote the expression of antioxidant enzyme-related genes, but also regulate the expression of potassium channel protein genes by regulating ROS signalling and activating CBL1-CIPK23 pathway, thus promoting plant nutrient absorption (Li *et al.* 2016).

Nevertheless, little has been known about how melatonin influences soybean growth under different nitrogen levels including the detailed regulatory mechanism of melatonin on nitrogen absorption and metabolism of soybean under different nitrogen levels. Further, it is unknown whether the regulatory pathway of melatonin is different between conditions of nitrogen deficiency and excess. The small root nodules could usually be seen on the main root after Vegetative 3 stage, indicating that the nitrogen required for Vegetative 1 (V1) stage mainly depends on exogenous supply (Purcell *et al.* 2014; Zeng *et al.* 2019). Therefore, sand culture treatments of soybean at seedling stage were conducted at low, normal, and high nitrogen levels (1.5, 7.5, and 15 mM, respectively), with and without 100 μ M melatonin application at V1 stage, to investigate the effect of nitrogen metabolism and soybean growth under different nitrogen levels. Thus, an innovative solution and theoretical basis to alleviate the effect of inappropriate nitrogen use on soybean seedlings' growth in the process of cultivation were provided.

Materials and methods

Plant materials and experimental treatments

Pots with a diameter of 20 cm and a height of 17 cm was used in the study. A layer of yarn net was laid on the pot bottom to prevent the roots from growing out of the basin. Then each pot was filled with 6 kg of river sand that had been previously washed once with tap water and twice with distilled water. Soybean variety 'Suinong 26' was used as experimental material. Six full seeds, uniform in colour and size, were sown in each pot, and then three uniform seedlings were retained in each pot at the Vegetative-Cotyledon (VC) stage. The tested plants were cultivated in the outdoor research base of the National Coarse Cereals Engineering Research Center in Daqing City, Heilongjiang Province, China (46°59'N, 125°17'E), during mid-June. The study area has a temperate monsoon climate.

From sowing to full expansion of the opposite leaves, 500 mL distilled water was sprinkled once a day to each plant. After the opposite leaves were unfolded, they were divided into three groups. Once a day, 500 mL 1/2 Hoagland nutrient solution was provided to the plants, with ammonium

sulfate as the only nitrogen source (see Supplementary Table S1 available at the journal website for the formula of other required nutrients) (Guan *et al.* 2016). Corresponding to low, normal, and high nitrogen levels, the nitrogen concentrations in the nutrient solution were 1.5 mM, 7.5 mM (corresponding nitrogen concentration in 1/2 Hoagland's nutrient solution), and 15 mM, respectively (Xia *et al.* 2017). To avoid salt accumulation in sand culture, 500 mL tap water was slowly drenched twice every 3 days. When soybean reached V1 stage, each treatment was randomly divided into two groups to spray with 100 μ M melatonin or distilled water, respectively, for 3 days at 9:00 pm. Six treatments were obtained: (1) LN (low nitrogen); (2) LN + MT (low nitrogen + 100 μ M melatonin); (3) CK (normal nitrogen); (4) CK + MT (normal nitrogen + 100 μ M melatonin); (5) HN (high nitrogen); (6) HN + MT (high nitrogen + 100 μ M melatonin).

After melatonin application for 24 h, three samples of functional leaves and three samples of roots were immediately placed in liquid nitrogen and then stored in a freezer at -80°C for gene expression analysis. The whole plant samples were taken at 9:00 am on the 6th, 12th, 18th, and 24th day after melatonin spraying to determine morphology, dry matter, total nitrogen, and urea content. The functional leaf samples were directly determined or wrapped in tin foil and stored in a freezer at -20°C for corresponding physiological and biochemical analysis.

Determination method of index

Measurement of morphological index and the accumulation of dry matter

The entire plant was washed with distilled water, and the plant height, the root length, the stem diameter, and the number of root nodules per plant were measured by conventional methods (Li *et al.* 2018). Further, the total leaf area of a single plant was measured by the Yaxin-1241 leaf area meter. Then the root, stem, and leaf were dried at 105°C for 0.5 h to inactivate the enzyme and then dried to constant weight at 80°C for about 48 h. The dried samples will be utilised to determine the dry matter content.

Measurement of nitrogen assimilating enzymes activity

Nitrate reductase (NR: EC 1.7.1.3) activity was measured according to the method of Sherrard and Dalling (1979). Four samples of 0.5 g fresh, functional leaves were weighed quickly for each treatment. One leaf was taken as a control, and the other three were used for activity determination. First, 1 mL 30% (m/v) TCA was added to the control, followed by 5 mL phosphate buffer solution (0.1 M, pH 7.5). Second, 5 mL potassium nitrate solution (0.2 M) was added

to each bottle to avoid light for 30 min and incubated at 25°C for 30 min. Subsequently, 1 mL 30% (m/v) TCA was added to stop the reaction in all bottles except in the control. The 2 mL of the reaction solution and 8 mL of nitric acid reagent were added to the test tube, and the colour was developed at room temperature for 20 min. The optical density of the supernatant was measured at 540 nm.

The determination method for glutamine synthetase (GS: EC 6.3.1.2) enzyme activity was according to Zhang *et al.* (2016). We ground 0.1 g samples into a powder with liquid nitrogen, added an 8 mL extract (100 mM Tris-HCl, 0.5 mM EDTA, 5 mM β -mercaptoethanol, pH 7.5) to it, and then centrifuged in 15 000g at 4°C for 20 min. The supernatant was used to determine the enzyme activity. A mixture of 1.6 mL of the reaction solution and 0.6 mL of the enzyme extract was incubated in a 25°C water bath for 5 min and 0.2 mL of hydroxylamine reagent was added to start the reaction. After 15 min of incubation, 1 mL of FeCl_3 reagent was added to terminate the reaction. The mixed solution was centrifuged in 15 000g for 10 min, and the optical density of the supernatant was measured at 540 nm.

The method for preparation of the extract to determine glutamate synthase (GOGAT: EC 1.4.7.1) and glutamate dehydrogenase (GDH: EC 1.4.1.2) activity was the same as that of GS. GOGAT activity was measured with L-glutamine to initiate the reaction, and one extinction value was measured every 30 s at 340 nm, 10 consecutive times. The enzyme activity was measured when the optical density decreased steadily. GDH activity was determined by adding 0.1 mL enzyme solution to activate the reaction. The decreasing value in absorbance after 3 min was recorded at 340 nm (Lin and Kao 1996; Singh *et al.* 2020).

Measurement of antioxidant indexes

The above frozen samples were used to determine the activities of superoxide dismutase (SOD), guaiacol peroxidase (GPX) and catalase (CAT) by the nitrogen blue tetrazolium reduction, guaiacol, and ultraviolet absorption methods, respectively (Zhang *et al.* 2017a). The content of malondialdehyde (MDA) formed due to membrane lipid peroxidation was determined by thiobarbituric acid heating colorimetry (Nakano and Asada 1981).

Measurement of inorganic nitrogen content

Fresh leaf samples (1 g) were macerated in liquid nitrogen, and the metabolites were extracted for 24 h with 10 mL of a methanol:chloroform:water (12:5:3, v:v:v) solution. After centrifugation at 2000g for 30 min, one volume of chloroform and 1.5 volume of water were added to each of the four volumes of the obtained supernatant. After phase separation over a 24 h period, the aqueous phase was collected. The resulted extract was concentrated for 15 h at 37°C and then used for biochemical analysis (Oliveira *et al.*

2013). NO_3^- was determined spectrophotometrically at 410 nm by the salicylic acid method described by Cataldo et al. (1975). The determination of NO_2^- was determined according to the method described by Hageman and Reed (1980).

Measurement of total nitrogen and ureide content

Nitrogen concentrations of plant tissues were measured using the Kjeldahl method. The dried samples of root, stem, and leaf were ground, and subsamples weighing about 0.1 g were digested with $\text{H}_2\text{SO}_4\text{--H}_2\text{O}_2$. Then the nitrogen content was determined using a Kjeldahl nitrogen meter (Zhang et al. 2016). The content of ureide in the root, stem, and leaf of the soybean tissue was determined according to the method used by Zhang et al. (2018).

Total RNA extraction and gene expression (qRT-PCR) analysis

Total RNA of functional leaf or root tip sample (50–100 mg) was extracted using an Axygen reagent kit (AxyPrep) and reverse-transcribed according to manufacturer's instructions (Prime Script RT Enzyme Mix I for TB Green qPCR, Takara). The gene-specific primers were designed with Primer Premier 5.0 (Table S2), and qRT-PCR was performed using a 10 μL mixture (5 μL TB Green Premix Ex Taq II; 3 μL distilled water; 0.5 μL PCR Forward Primer; 0.5 μL PCR Reverse Primer; 1 μL cDNA) according to manufacturer instructions (CFX96 Real-Time PCR Detection System, Takara). A two-step PCR method was used with the following conditions: pre-denaturation at 95°C for 30 s, 40 cycles at 95°C for 5 s, and 60°C for 30 s. The specialist software Bio-Rad iQ5 was used to collect data. Actin (gene ID: *Glyma18g52780*) was adopted as an internal reference for the normalisation of gene expression. In the qRT-PCR analysis, samples of two plants from per pot treated with melatonin for 24 h were mixed as a single replicate, three pots per treatment were considered as three biological replicates. Each biological replicate had three technical replicates.

Three repetitive values were recorded in the determination process of the above physiological, biochemical, and morphological indexes. Each value was the average value of three mixed samples in the same container.

Statistical analyses

The experiment was conducted in a completely randomised design. The data obtained were analysed by Microsoft Excel 2016. l.s.d. and Duncan's multiple range test ($P \leq 0.05$) were performed using the statistical program SPSS 20.0. Finally, charts including the principal component analysis (PCA) biplot were formed with Origin 2017 software.

Results

Effects of exogenous melatonin on plant morphological parameters

According to the phenotype analysis of four time points (Fig. 1, Table S3), it was evident that, compared with the control, the plant height, stem diameter, total leaf area, and dry matter accumulation of seedlings were inhibited under low nitrogen level. The growth of seedlings under high nitrogen levels was also significantly inhibited. On the 24th day after treatment began, the dry matter accumulation at a high nitrogen level was significantly less than that of the control by 18.41%. The application of melatonin promoted the growth of seedlings in different degrees under different nitrogen concentrations. With the establishment of plant morphology, it was observed that on the 24th day after melatonin treatment, the stem diameter and root length of seedlings increased significantly at all nitrogen levels. However, there was no significant difference in plant height between melatonin sprayed and unsprayed melatonin treatments. In addition, the application of melatonin significantly increased the total leaf area per plant at low N and normal N levels.

The results of the root nodule analysis indicated that melatonin promoted root nodule growth and increased the average dry weight of root nodules per plant under low nitrogen levels. In contrast, the average dry weight of a single root nodule increased significantly under high nitrogen levels with melatonin. From the analysis of the dry weight of the aboveground and underground parts, it was concluded that the application of melatonin further increased the dry matter accumulation of all parts under low and normal nitrogen levels. However, it mainly promoted root development under high nitrogen treatment so that the root:shoot ratio increased by 10.99% compared to that under HN. Finally, compared to soybeans without melatonin treatment, the total dry matter accumulation per plant of soybeans exposed to melatonin at low N, normal N, and high N levels increased significantly by 9.80%, 14.06%, and 11.44%, respectively.

Effects of exogenous melatonin on the activity of enzymes related to nitrogen assimilation

On the whole, the activities of enzymes related to N metabolism were significantly lower than those of control due to low N level. As the N level was higher than control, the activities of these enzymes tended to be enhanced in a short period. However, with the extension of high N supply time, the activities of these enzymes decreased gradually compared with the previous high N level. On the 24th day after treatment began, no significant difference was shown observed in the activities of the GS and GOGAT between the high N and control group, and the GDH activity in high N

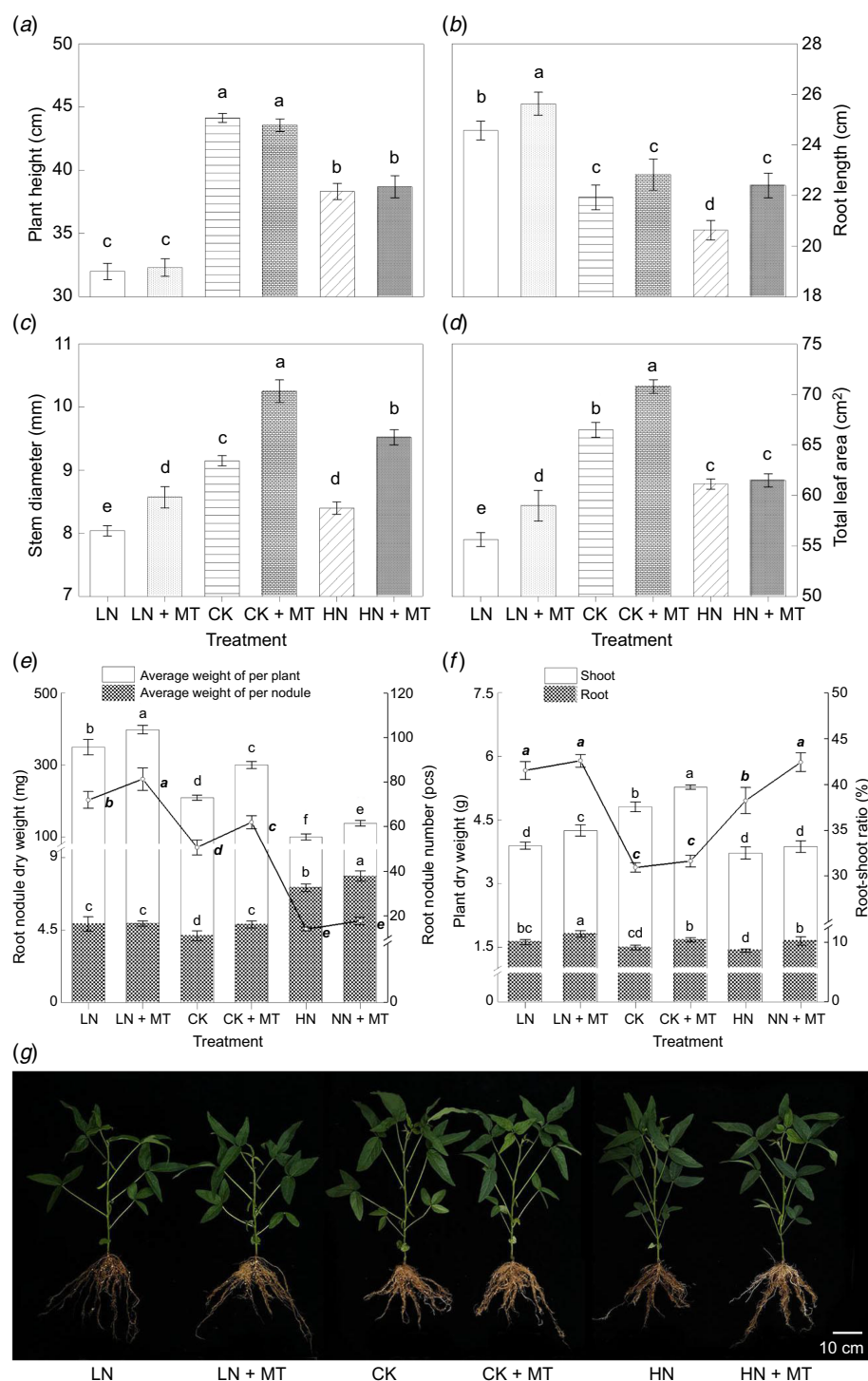


Fig. 1. The effect of exogenous melatonin on soybean plant height (a), root length (b), stem thickness (c), total leaf area (d), root nodule dry weight (average weight of per plant and per nodule, column chart) and number of individual plant (line chart) (e), shoot and root dry weight (column chart), root:shoot ratio (line chart) (f) and phenotypic response (g) 24 days after spraying under different nitrogen levels. LN, low nitrogen (1.5 mM); LN + MT, low nitrogen (1.5 mM) + 100 μ M melatonin; CK, normal nitrogen (7.5 mM); CK + MT, normal nitrogen (7.5 mM) + 100 μ M melatonin; HN, high nitrogen (15 mM); HN + MT, high nitrogen (15 mM) + 100 μ M melatonin. The values are presented as mean $\pm$ s.e. ($n = 3$). Different letters in one measuring group indicate statistically significant differences at $P \leq 0.05$.

group was lower than that of control (Fig. 2d). Notably, the effects of melatonin on different enzymes activities were distinct.

Compared with low N alone, melatonin significantly increased the NR activity under low N levels at 6th and 12th day after spraying, which improved by 50.08% and 25.74%, respectively. Nevertheless, the activity was inhibited after melatonin treatment in the control and high N group. There were no apparent differences in NR activity between two groups with and without melatonin on the 24th day after melatonin (Fig. 2a).

Through the analysis of GS activity (Fig. 2b), it could be concluded that melatonin could enhance its activity at various N levels, which were higher than that without melatonin. When treatment days of melatonin increased to 12, the activity of GS in the LN + melatonin group was even higher than the control. Compared with the control, the CK + melatonin group was also significantly increased by 20.80% and 20.84%, respectively, on the 12th and 18th day after melatonin treatment. Notably, the promotion effect of

melatonin under high N was the most obvious on the 18th day after spraying, which was 19.05% higher than that of high N alone. The contribution of melatonin to GOGAT and GDH activity was closely similar to its effect on GS (Fig. 2c, d). These enzymes activity of leaf with melatonin under low N level was remarkably improved. In addition, this promoting effect was evident on the 12th day after melatonin treatment in the groups fed with normal N and high N.

Effects of exogenous melatonin on the activity of antioxidant enzymes and the content of malondialdehyde

As a major indicator of the degree of membrane lipid peroxidation, the content of MDA was higher than that of the control (Fig. 3d) in the early stage of high N treatment. The application of melatonin could significantly promote SOD, GPX, and CAT activities under high N levels (Fig. 3a–c), and decreased the content of MDA. In the high N treatment,

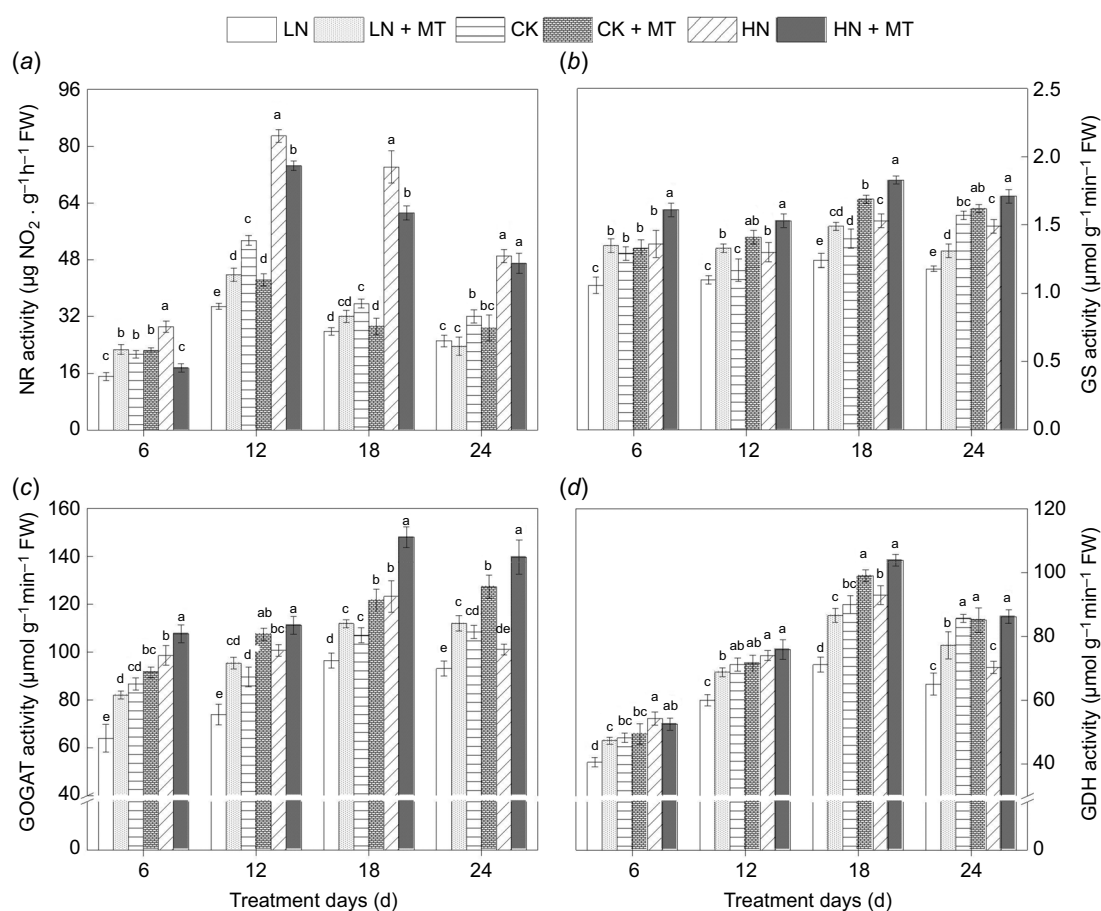


Fig. 2. Effects of exogenous melatonin on the activities of nitrate reductase (NR, a), glutamine synthetase (GS, b), glutamate synthase (GOGAT, c) and glutamate dehydrogenase (GDH, d) in plant leaves under different N levels. The abbreviated meaning of each treatment is the same as in Fig. 1. The values are presented as mean ± s.e. (n = 3). Different letters in one measuring group indicate statistically significant differences at $P \leq 0.05$.

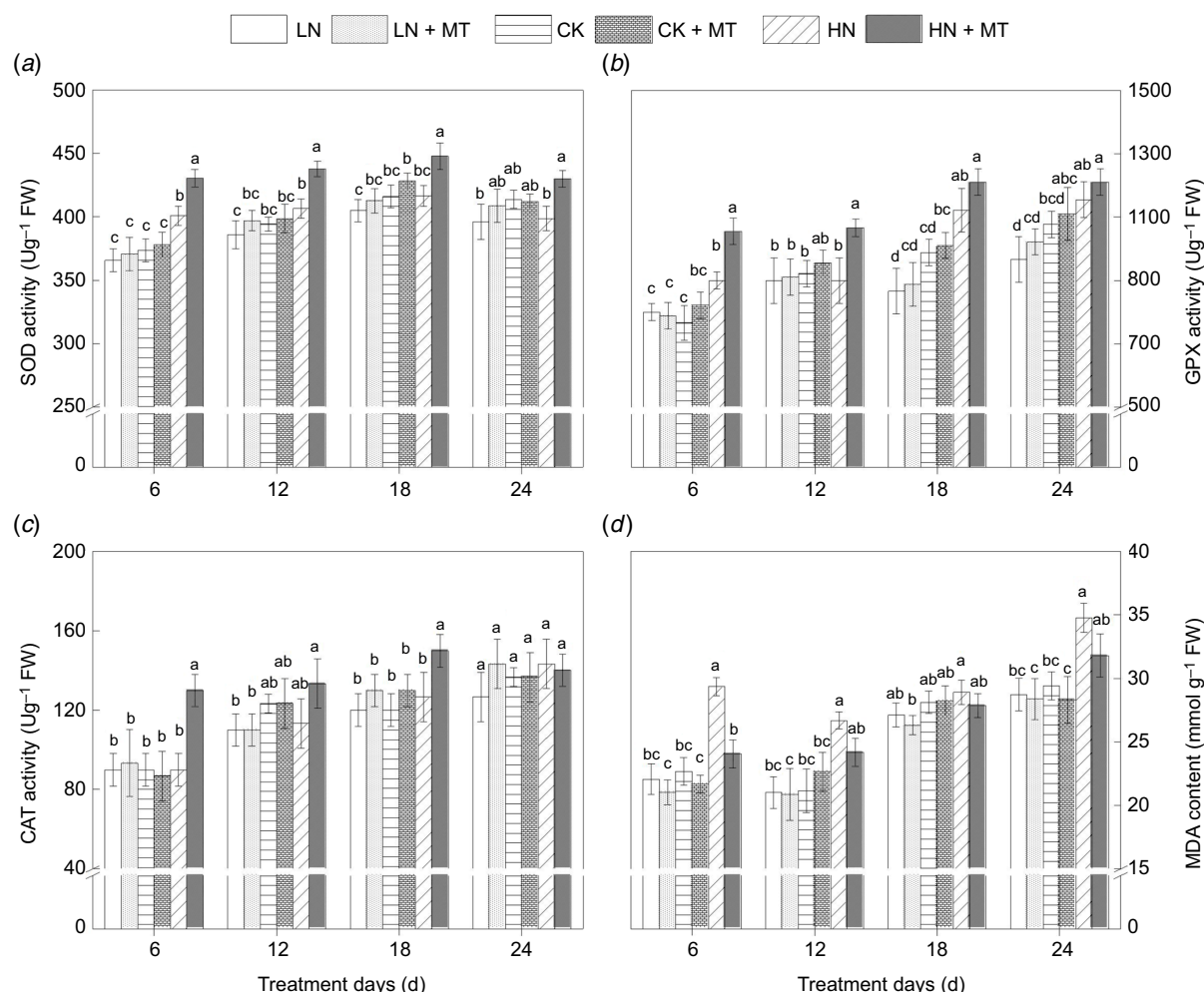


Fig. 3. The effect of exogenous melatonin on the activity of superoxide dismutase (SOD, a), guaiacol peroxidase (GPX, b), catalase (CAT, c) and the content of malondialdehyde (MDA, d) in leaves of plants under different N levels. The abbreviated meaning of each treatment is the same as in Fig. 1. The values are presented as mean $\pm$ s.e. ($n = 3$). Different letters in one measuring group indicate statistically significant differences at $P \leq 0.05$.

MDA content returned to the control level after melatonin spraying. However, compared with the control, there was rarely a significant distinction in antioxidant enzymes activity and MDA content between the low N and the control.

Effect of exogenous melatonin on nitrate and nitrite content in functional leaves

Combined with the analysis of nitrate-nitrogen (Fig. 4a) and nitrite content (Fig. 4b) in functional leaves, it was found that levels of the two inorganic nitrogens were significantly lower than those of the control at low N levels. However, the application of melatonin at this nitrogen level significantly promoted the absorption of nitrate. Thus, nitrite accumulation increased, decreasing the difference between the low N treatment and the control (Fig. 4a). However, under both normal N and high N concentrations, the application of

melatonin showed negative feedback regulation on the accumulation of nitrate, and the content of nitrite in leaves treated with melatonin was generally lower. The inhibitory effect on nitrate-nitrogen was significant on the 12th and 24th days after melatonin treatment.

Effects of exogenous melatonin on total nitrogen content of each tissue

The nitrogen content of soybean leaves, stems, and roots increased with the increase in exogenous N concentration (Fig. 5). Results demonstrated that melatonin modulated the nitrogen accumulation under low N and normal N levels, mainly reflected in leaves and roots. Nevertheless, there was rare discrimination in nitrogen content at high N levels between the two groups with or without melatonin.

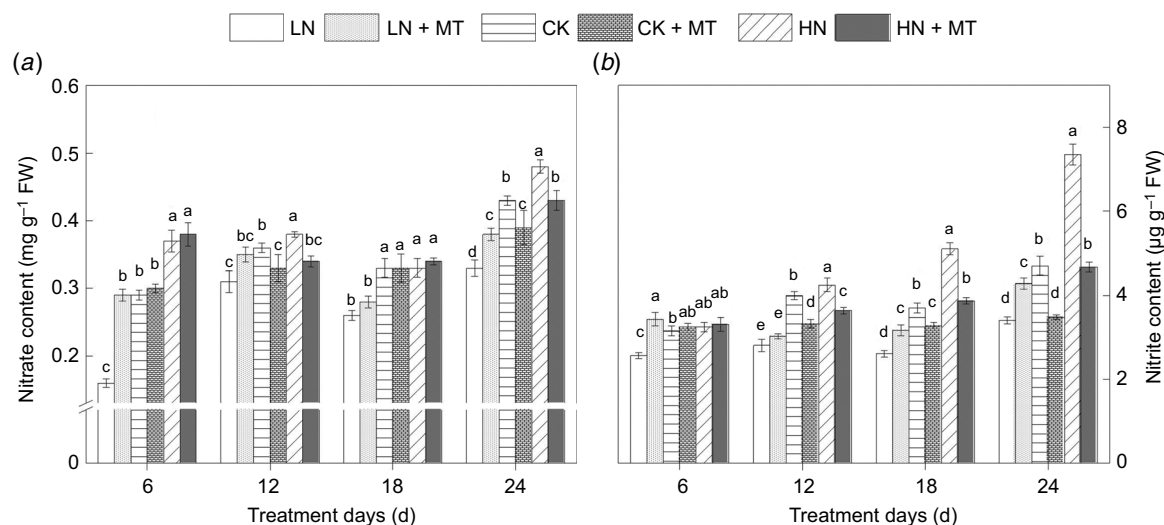


Fig. 4. The effect of exogenous melatonin on the content of nitrate (a) and nitrite (b) in leaves of plants under different N levels. The abbreviated meaning of each treatment is the same as in Fig. 1. The values are presented as mean $\pm$ s.e. ($n = 3$). Different letters in one measuring group indicate statistically significant differences at $P \leq 0.05$.

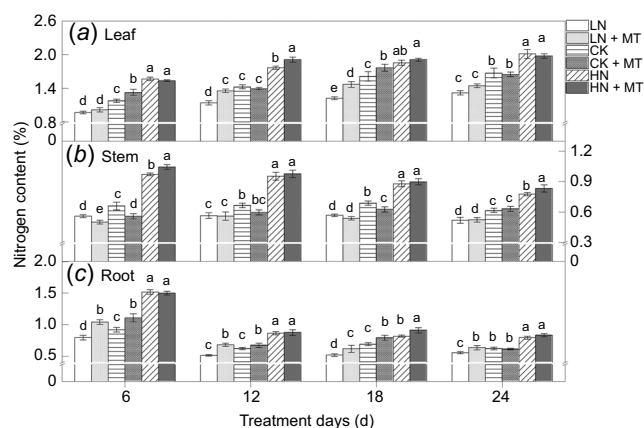


Fig. 5. Effects of exogenous melatonin on nitrogen content of leaves (a), stems (b) and roots (c) of plants at different N levels. The abbreviated meaning of each treatment is the same as in Fig. 1. The values are presented as mean $\pm$ s.e. ($n = 3$). Different letters in one measuring group indicate statistically significant differences at $P \leq 0.05$.

Effects of exogenous melatonin on ureide content of each tissue

It can be seen that the ureide content of the soybean seedling stage was very limited, but that it increased with the enhancement of nitrogen fixation ability (Fig. 6). Melatonin played a key role in promoting the accumulation of ureide content in various tissues of soybean at low N concentrations. Further analysis concluded that the addition of N supply would inhibit ureide accumulation, alleviating effectively with melatonin. The contribution of melatonin to acylurea accumulation at normal N and high N levels was mainly reflected in stems and roots.

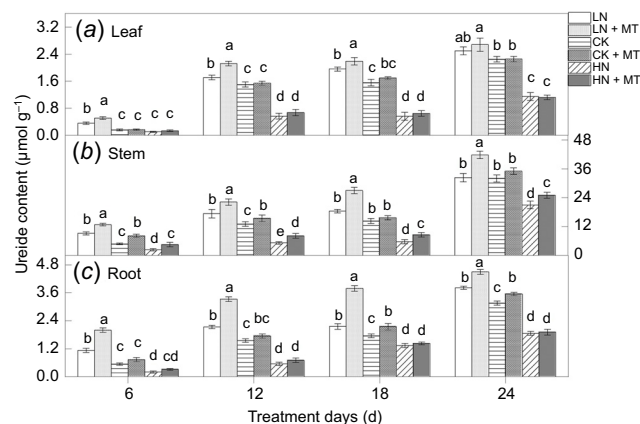


Fig. 6. Effects of exogenous melatonin on ureide content of leaves (a), stems (b) and roots (c) of plants at different N levels. The abbreviated meaning of each treatment is the same as in Fig. 1. The values are presented as mean $\pm$ s.e. ($n = 3$). Different letters in one measuring group indicate statistically significant differences at $P \leq 0.05$.

Effects of exogenous melatonin on the expression of genes related to nitrogen metabolism

To investigate the molecular mechanism for melatonin-induced nitrogen stress tolerance, we examined the effects of melatonin on gene expression related to nitrogen metabolism (Fig. 7). Through qRT-PCR, it was found that under low nitrogen treatment, the gene expression of the ammonium transporter gene (*AMT1*, *AMT2*), nitrite reductase gene (*NiR*), glutamine synthetase gene (*GS1β*), glutamate synthase gene (*GOGAT*), and amino acid transport gene (*AAT*) in leaves and roots was significantly upregulated under

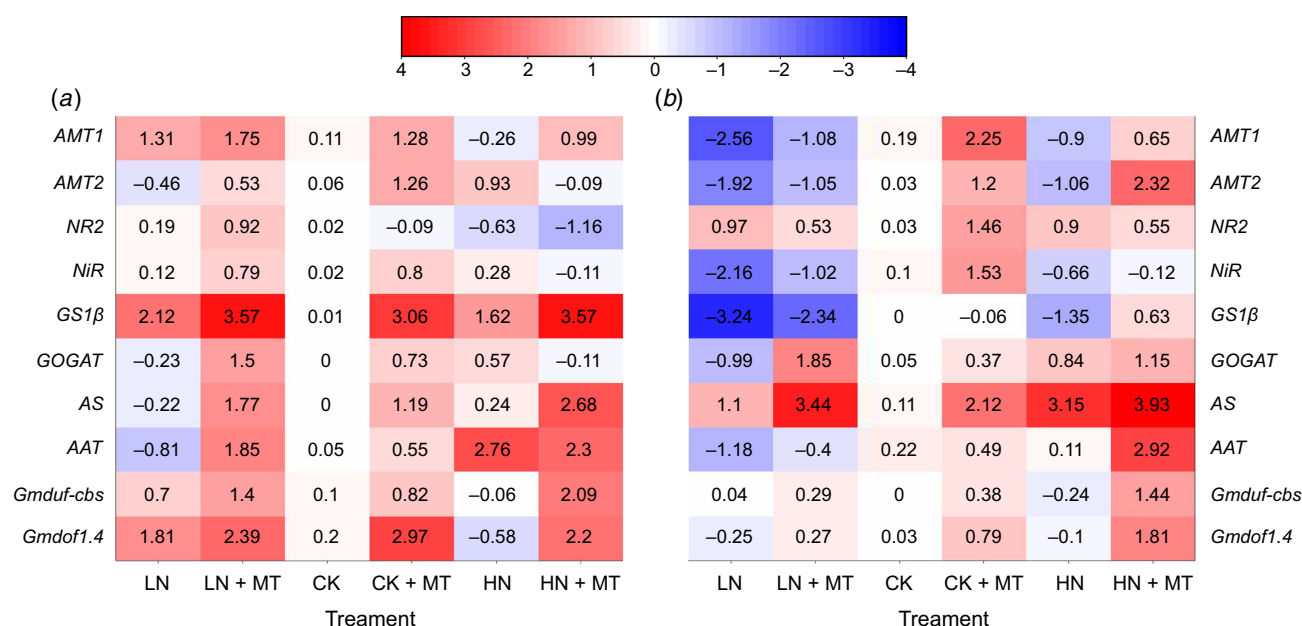


Fig. 7. Relative expressions of ammonium transporter gene (*AMT1*, *AMT2*), nitrate reductase gene (*NR2*), nitrite reductase gene (*NiR*), glutamine synthetase gene (*GS1β*), glutamate synthase gene (*GOGAT*), asparagine synthase gene (*AS*), transmembrane amino acid transporter protein (*AAT*), and genes related to resisting abiotic stress (*Gmduf-cbs*) and regulating photosynthetic carbon assimilation (*Gmdof1.4*) of leaf (a) and root (b). The abbreviated meaning of each treatment is the same as in Fig. 1. Take the average value of the actin gene and CK expression as reference, the values were the average of $2^{-(\Delta\Delta CT)}$ presented.

the stimulation of melatonin. In addition, the nitrate reductase gene (*NR2*), which catalysed the reduction of nitrate to nitrite, was significantly upregulated in leaves. Under high N conditions, melatonin application stimulated the upregulation of genes (*AMT1*, *AMT2*, *NiR*, *GS1β*, *GOGAT*, *AS*, *AAT*) involved in nitrogen assimilation, which was mainly reflected in roots. However, the expression of *NR2* was regulated by melatonin negative feedback in both leaves and roots under this condition. In addition, *Gmduf-cbs* and *Gmdof1.4* genes were also significant, and their expression levels are all upregulated compared with treatments without melatonin spraying at three nitrogen levels.

Effect of exogenous melatonin on all the parameters studied under different nitrogen levels

Different treatments and determination indicators were merged in a single PCA biplot graph to visualise further (Fig. 8). The PCA biplot for 6th, 12th, 18th and 24th day after melatonin treatment explained 70.9%, 71.4%, 72.8%, and 68.7% of the total variability, respectively. Among them, PC1 accounted for 50.2%, 51.5%, 47.5%, and 38.3%, respectively, and PC2 accounted for 20.7%, 19.9%, 25.3%, and 30.4%, respectively. Moreover, from the score plot of different treatments at four sampling time points, it could be observed that the distance between LN + MT and CK was much smaller than that between LN and CK, and similarly, the

distance between HN + MT and CK was much less than the distance between HN and CK. In addition, the factor loading plot reflects that most of the relevant indicators of analysis were discriminating traits that could significantly distinguish the differences between treatments, i.e. their vectors on the biplot were all relatively long. Therefore, the index studied could reflect that melatonin treatment was beneficial in reducing the gap between the control and low or high nitrogen conditions on plant development level and nitrogen metabolism.

Discussion

Melatonin is a pleiotropic molecule with many diverse actions in plants (Shi *et al.* 2015; Arnao and Hernández-Ruiz 2019). Although it had been confirmed that exogenous melatonin has a certain regulatory effect on nitrogen metabolism under many treatment conditions (Turk and Erdal 2015; Arora and Bhatla 2017; Zhang *et al.* 2017b), there was not enough data to prove whether exogenous melatonin could enhance the nitrogen adaptability of soybean by regulating nitrogen cycles. Therefore, our study was the first to demonstrate the mechanism of melatonin-regulated nitrogen metabolism in soybean under different nitrogen levels.

Under low N levels, the nitrogen metabolism of soybean was inhibited; however, the application of melatonin could significantly accelerate the absorption and utilisation of

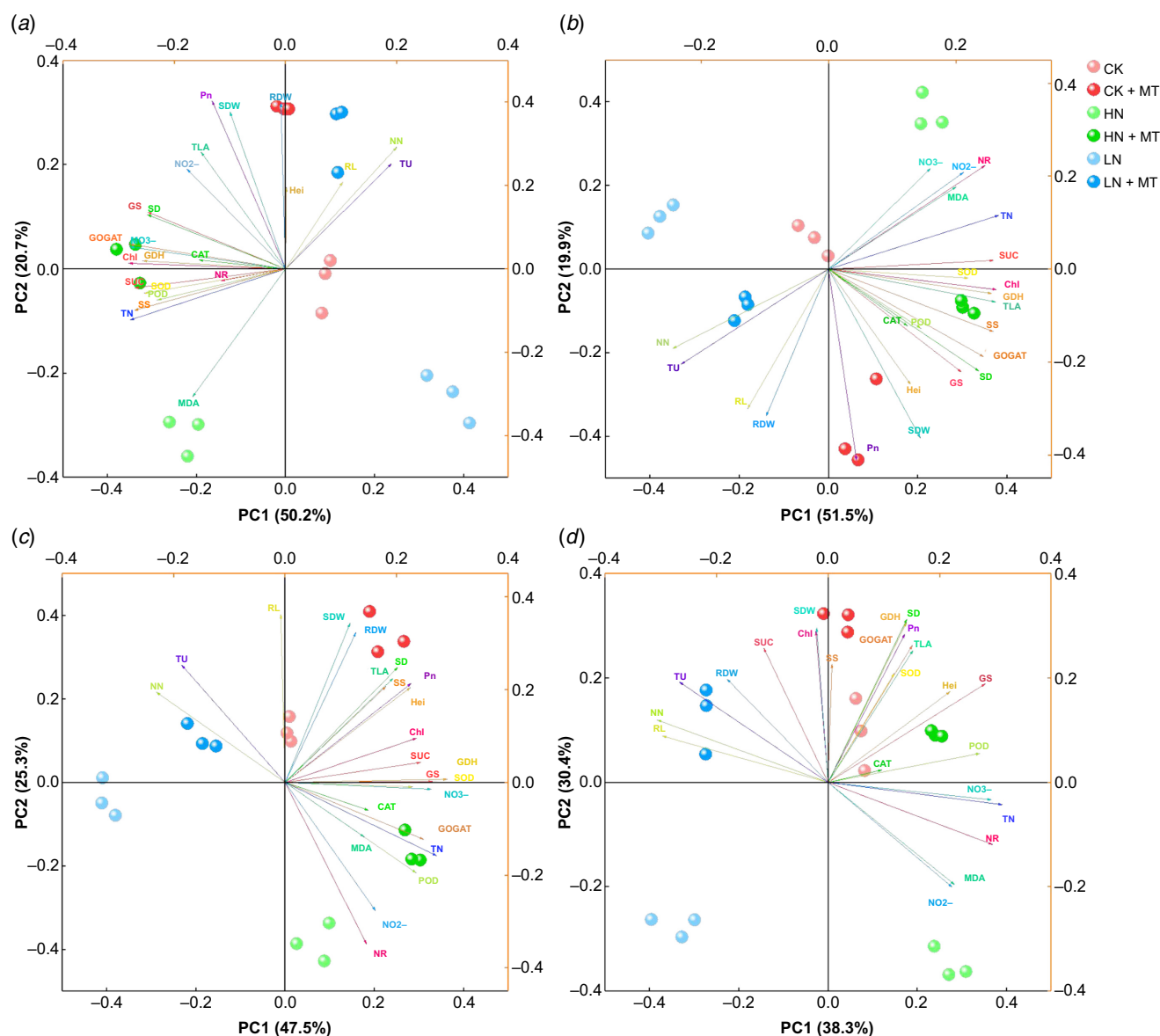


Fig. 8. Principal components analysis (PCA) biplot showing the relationships between crops harvested from soybean sprayed with melatonin at different nitrogen levels and related indicators at 6th (a), 12th (b), 18th (c) and 24th (d) day after spraying melatonin, describing their nitrogen metabolism and growth characteristics. The relevant indicators were indicated by arrows, while the different treatments were indicated as points. NR, nitrate reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase; SOD, superoxide dismutase; POD, guaiacol peroxidase; CAT, catalase; MDA, malondialdehyde; NO_3^- , nitrate; NO_2^- , nitrite; TN, total nitrogen; TU, total ureide; Pn, net photosynthetic rate; Chl, chlorophyll; SUC, sucrose; SS, soluble sugar; Hei, plant height; RL, root length; NN, nodule number; SD, stem diameter; TLA, total leaf area; SDW, dry weight of the shoot; RDW, dry weight of the root. The abbreviated meaning of each treatment is the same as in Fig. 1.

nitrogen by seedlings under this condition. Our study found that the expression of ammonium transporter genes (*AMT1*, *AMT2*) was significantly upregulated at this nitrogen level after melatonin was applied. It has been well documented that *AMT1* and *AMT2* proteins play a critical role in ammonium uptake and root-to-shoot translocation, respectively (Gu et al. 2013; Giehl et al. 2017; Chen et al. 2019). Therefore, melatonin could stimulate ammonium uptake of seedlings to compensate

for the nitrogen demand of seedlings. However, because NR is greatly affected by light intensity, some studies have pointed out that its activity would be significantly inhibited in dark conditions (Shaha 2003; Creighton et al. 2017). Therefore, most of the nitrate absorbed by the root was transported to its leaves and then assimilated by NR, as reported by many studies (Timpo and Neyra 1983; Pinto et al. 2014). Additionally, our study proved that the upregulation effect of

melatonin on NR gene expression was mainly in functional leaves. Further, it was also found that the accumulation of nitrate-nitrogen in functional leaves returned to the control level 6–12 days after melatonin treatment. However, the activity of nitrate reductase, which catalysed its reduction, significantly increased. This fully reflected that melatonin was also beneficial to nitrate absorption by soybean at low N levels (Fig. 4). Previous studies on wheat under low N stress showed that melatonin-treated wheat maintained higher nitrate levels and total nitrogen content. The activities of NR and GS were elevated (Qiao *et al.* 2019), which was similar to that observed in our study. The results of this study summarised that: (1) melatonin was beneficial to nitrogen absorption by soybeans at low nitrogen levels; (2) melatonin could promote nitrogen assimilation by upregulating the expression of enzyme-encoding genes, such as glutaminase and glutamate synthase, which were involved in ammonium assimilation; and (3) melatonin enhanced the activity of the aforementioned enzymes. All kinds of amino acids synthesised by the nitrogen metabolism pathway will be transported to specific sites through the amino acid transporter in the cell (Cheng *et al.* 2016). Therefore, the results of upregulation of melatonin-stimulated amino acid transporter gene (*Glyma11g11310*) in this study also indicated that melatonin played a unique role in enhancing nitrogen use efficiency.

In this study, the effect of high nitrogen treatment on soybean seedlings was consistent with the result that excessive nitrogen supply was harmful to plant growth, as demonstrated by Kong *et al.* (2017). Previous studies have pointed out that this adverse effect was likely caused by oxidative stress from the excessive accumulation of ROS (Skopelitis *et al.* 2006). In this study, the analysis of MDA content revealed that it was found that its content at high N levels was significantly higher than that of the control (Fig. 3d). It was clear that the excessive accumulation of MDA was closely correlated with the toxicity caused by excessive nitrogen application (Bi *et al.* 2007). As a free radical scavenger, melatonin has been proved to significantly promote the activity of antioxidant enzymes at high N levels (Fig. 3a–c), similar to the response mechanism of melatonin in alleviating nitrogen stress summarised by previous studies (Wang *et al.* 2016). This is consistent with the view that melatonin plays a significant role in plant stress signals (Gong *et al.* 2017). And it was considered a key component in the center of the redox network from which the different biochemical, cellular, and physiological responses were controlled (Arnao and Hernández-Ruiz 2018, 2019). Therefore, melatonin is predicted to alleviate the harmful effects of high N levels on soybean growth by promoting the activities of antioxidant enzymes, inhibiting the accumulation of RNS and ROS, thus reducing the degree of membrane lipid peroxidation.

In addition, in recent years, proteins containing cystathionine- β -synthase (CBS) domains have been considered to be related to abiotic stress resistance. This family plays an essential role in plant responses to abiotic and abiotic

stresses (Hao *et al.* 2011). Among them, there are many stress signaling-related sites in the upstream promoter region of *Gmduf-cbs* gene. The upregulation of this gene has been proved to alleviate nitrogen stress (Kushwaha *et al.* 2009; Hao *et al.* 2021). Therefore, the result of significant upregulation of *Gmduf-cbs* gene after melatonin application in this study provided a novel opinion for promoting soybean nitrogen adaptability. Still, the specific response pathway is necessary to be determined.

This study also revealed that under the condition of excessive nitrogen supply, exogenous melatonin was also beneficial to prevent the excessive accumulation of ammonium ions in mesophyll and root cells. Due to excessive nitrogen supply, plant development was significantly inhibited, mainly in the root (Chen *et al.* 2021). Therefore, in this study, it was observed that the expression of key genes about nitrogen metabolism was alleviated in melatonin treatment, and the upregulation effect was more evident in the root. The expression of GS and other genes that catalyse further assimilation of ammonium ions were increased, and the protein activity of GS also enhanced, which were consistent with the regulation pathway of soybean under low N condition. Zhang *et al.* (2017b) also concluded a similar conclusion in the mechanism of melatonin alleviating excessive nitrogen stress of cucumber. Moreover, melatonin was confirmed to significantly reduce nitrate accumulation in lettuce leaves and roots (Zhou *et al.* 2019). In this study, in addition to the above similar conclusions, it was also observed that melatonin could significantly inhibit the accumulation of nitrite in soybean functional leaves, thus alleviating the toxicity caused by excess nitrogen.

The results also showed that melatonin significantly upregulated the expression of *Gmdof1.4* gene, a member of *Dof1* family, under three nitrogen concentrations. Members of the *DOF* family were known to be involved in the tolerance of plants to nitrogen stress and improve plant growth. It has been suggested that genes carrying *DOF* domain function were related to the co-expression of organic acid metabolising enzymes (Yanagisawa *et al.* 2004; Hao *et al.* 2011). Therefore, it may be predicted that melatonin may enhance nitrogen metabolism by activating the organic acid pathway to produce the carbon skeleton needed for nitrogen assimilation (Massange-Sánchez *et al.* 2016).

Based on the hypothesis that melatonin promoted carbon metabolism, through the determination of chlorophyll content and net photosynthetic rate in functional leaves, it was confirmed that the photosynthetic capacity was indeed improved with melatonin treatment (Supplementary Fig. S1A, B). And compared with the control without melatonin treatment, the accumulation of soluble sugar of photosynthate also increased at all nitrogen levels (Fig. S1C, D). Previous studies had shown that melatonin could directly stimulate gene expression involved in photosynthesis (Wei *et al.* 2015). In addition, Zhang *et al.* (2018) had found that melatonin could potentially increase soybean PSII efficiency, improve leaf area

index, and increase dry matter accumulation under drought stress. And Erdal (2019) had also confirmed that melatonin could significantly promote C and N metabolism in maize, and the intuitive phenomenon was reflected in better morphological parameters. The coordination between carbon and nitrogen metabolism underlies the growth process in higher plants (Duan et al. 2018). Nitrogen metabolism provides a variety of amino acids for carbon metabolism, while photosynthetic reactions are involved in the synthesis, regulation, and the maintenance of the enzymes of nitrogen assimilation pathway, cellular respiration, in particular, the citric acid cycle, provides carbon skeletons for amino acid biosynthesis (Lawlor 2002). Therefore, the promotion of melatonin on soybean growth under an unsuitable nitrogen supply level is attributed to the promotion of nitrogen metabolism and its direct stimulation of the carbon metabolism pathway.

In this study, the analysis of plant morphological parameters also confirmed the positive effect of melatonin on the growth and development of soybean. It mainly manifested in the recovery of growth when the plant was treated with nitrogen deficiency or excess (Fig. 1, Table S3). It is worth while noting that melatonin could significantly alleviate the growth inhibition of soybean nodules, especially at high N levels. Since melatonin has been proved to significantly promote the effect on all root morphogenetic processes, it was believed that the improvement of nodules development might be related to the restoration of root growth (Erland and Saxena 2018). As one of the main storage forms of nitrogen, ureide is mainly produced by nodule fixation. And in the results of our study, the accumulation of ureide increased in varying degrees after melatonin treatment, which also proved the conclusion that melatonin is beneficial to root nodules. However, it

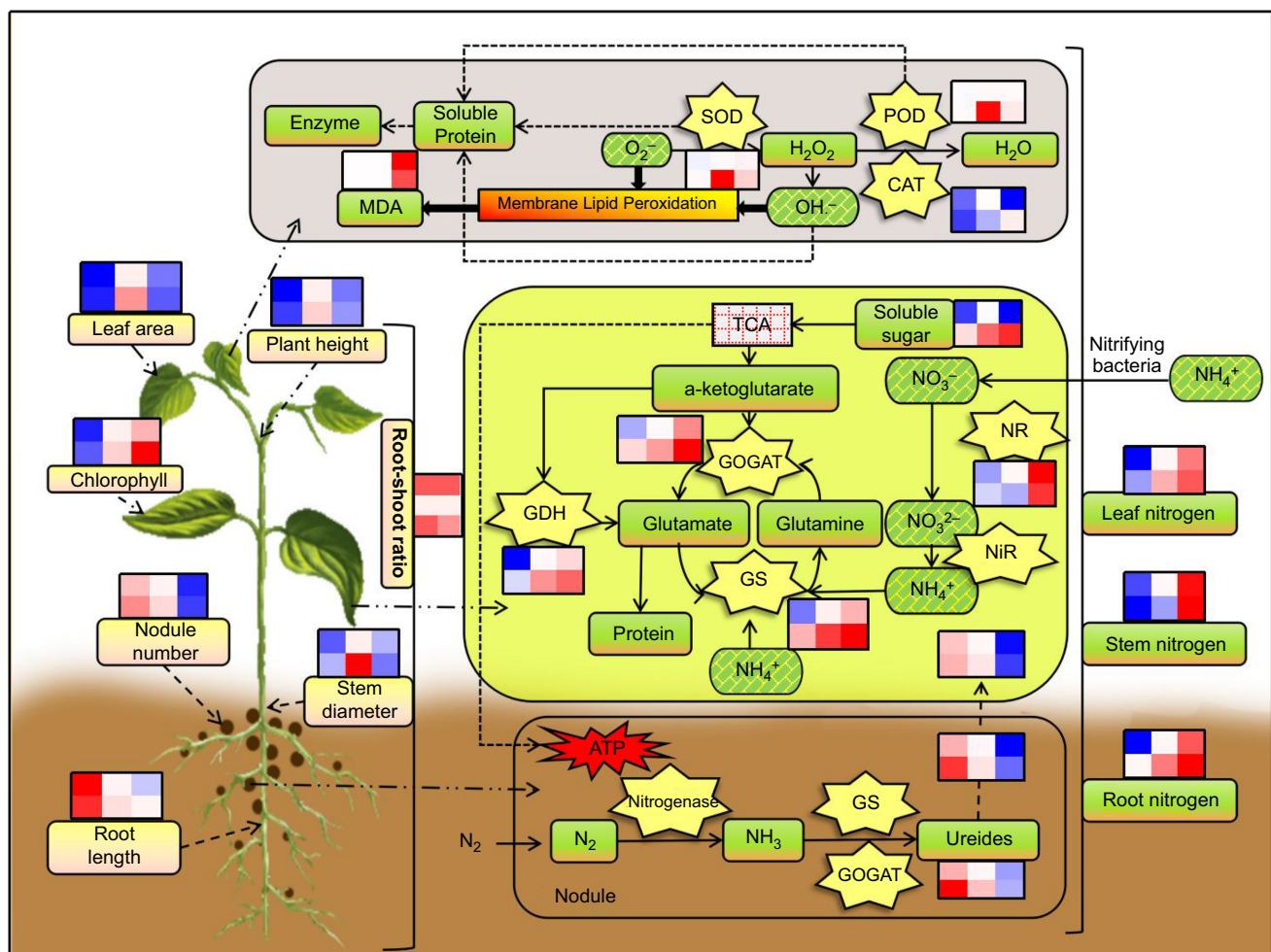


Fig. 9. Schematic model displayed exogenous melatonin on the physiological mechanism of soybean seedlings under three N levels. From top to bottom, the three parts represent antioxidant system, nitrogen metabolism system and nodule nitrogen fixation system, respectively. The square represented the material content index, and the heptagonal star represented the enzyme activity index. The six small colour blocks alongside of indexes represent the rising or falling level of the index compared with CK, and the upper three pieces from left to right indicate that the corresponding treatment is LN, CK, HN, while the treatment in the lower layer is LN + MT, CK + MT, HN + MT, respectively. The blue depth indicated the degree of decrease, and the red depth indicated the degree of increase.

needs to be researched deeply on the specific regulatory pathway of melatonin in promoting nodule growth.

In short, as shown in Fig. 9, nitrogen deficiency could directly limit the activity of nitrogen assimilation enzymes, reduce the synthesis of chlorophyll, limit photosynthetic capacity, thus affect plant dry matter accumulation and make plants yellowing and dwarfing. Melatonin could effectively promote nitrogen assimilation enzyme activity at low nitrogen levels, enhance nitrogen absorption and utilisation, increase chlorophyll content, and enhance photosynthetic carbon metabolism to alleviate the effects of insufficient nitrogen supply on soybean growth and yield. However, under high N levels, although the ability of nitrogen assimilation was enhanced quickly, the nitrogen fixation ability of root nodules was significantly inhibited. High N treatment led to increased membrane lipid peroxidation products and oxidative stress in plants (Skopelitis *et al.* 2006). As a result, other physiological and metabolic activities were disturbed, affecting the formation of soybean yield (Arnao and Hernández-Ruiz 2018, 2019). Nevertheless, the application of melatonin could enhance the assimilation of ammonium nitrogen, reduce the accumulation of inorganic nitrogen, alleviate the stress response caused by high nitrogen, and increase the formation of yield by increasing the activity of the antioxidant enzyme in the case of excessive nitrogen application.

Conclusion

This study confirmed that melatonin can promote the growth of soybean seedlings under different nitrogen concentrations. However, the regulatory effect of melatonin on various nitrogen levels of soybean was reflected in the different metabolic regulation pathways. At low N levels, it not only stimulated nitrogen absorption but also induced the upregulation of genes expression involved in nitrogen assimilation (*AMT1*, *AMT2*, *NR*, *NiR*, *GS*, *GOGAT*, *AS*, *AAT*) and the activity of several enzymes (*NR*, *GS*, *GOGAT*, *GDH*). Under high N levels, it was primarily conducive to the further assimilation of catalytic ammonium to reduce the accumulation of inorganic nitrogen in cells. It could significantly enhance the activity of antioxidant enzymes (*SOD*, *GPX*, *CAT*) to alleviate oxidative stress and other physiological damage. Results confirmed the effects of melatonin on the growth and nitrogen metabolism of soybean. They support an original idea and theoretical basis for understanding regulation of nitrogen use, and point to possible methods for use in remediation of insufficient or excessive nitrogen fertiliser application in soybean.

Supplementary material

Supplementary material is available [online](#).

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