Effects of melatonin on the sugar metabolism and antioxidative response to excess nitrate stress in lettuce seedlings

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Abstract. Water and soil pollution by nitrate has caused severe impact to environment, especially the inhibition of plant growth due to the excess nitrate stress. Although previous studies reported the melatonin could mitigate injury of plants in response to excess nitrate stress, the alleviation mechanism of melatonin is still unclear. In this study, lettuce seedlings were used as materials, and the main sugar content, sucrose synthesis and metabolism related enzymes as well as ROS metabolism were investigated. There were dramatic changes in sucrose, glucose and fructose content under excess nitrate stress. Exogenous melatonin further increased the sugar content mentioned above, and melatonin increased some sucrose synthesis and metabolism related enzymes under excess nitrate stress, including sucrose phosphate synthase and acid invertase, enhancing sucrose synthesis and metabolism ability. In addition, the content of malondialdehyde and hydrogen peroxide was increased by excess nitrate stress, while inhibited at presence of melatonin. The activities of SOD and POD were also increased by exogenous melatonin in response to excess nitrate stress. In a conclusion, it is suggested that there is a cross-talk between sugar and ROS scavenging pathway under excess nitrate stress, which is enhanced by melatonin, thus promoting the lettuce plants resistance to stress.

1. Introduction

In China, secondary salinization has become a threat to agriculture in protected cultivation. Excess nitrate stress induced inhibition of plant yield and severe oxidative damage in cucumbers [1] and tomatoes [2]. However, the mechanism of excess nitrate stress remains unclear. In addition, it is also necessary to solve the mitigation problem in response to excess nitrate stress.

Sugars are considered as both nutrients and important signaling molecule, which participate in plant growth, development and stress resistance. Both glucose and sucrose are prominent that control gene expression in plant resistance to stress [3]. Sucrose can be further metabolized to produce glucose and fructose. It is great interest in understanding the processes that are involved in sucrose and glucose participating in stress responses. More and more reports pointed out there was a cooperation of sugars and ROS scavenger system [3]-[5]. The soluble sugars play an important role in triggering the production of specific ROS scavengers against oxidative stress [4]. In addition, it was recently indicated that soluble sugars, especially when they are present at higher level, might scavenge ROS by themselves [6]. The putative roles of sugars and sugar-antioxidant network in plant cells would be beneficial to perfect plant stress resistance system.
Melatonin (MEL; N-acetyl-5-methoxytryptamine) is an indoleamine small molecular substance that has been shown to play important role in growth, development, and stress response of plants. Current evidence indicates that exogenous melatonin enhances stress tolerance in plants [7]. Melatonin was also found to cross talk with sugar-based signaling pathways under many processes of plant growth, the author found low concentration of melatonin promote the maize seedlings growth by enhancing the sugar metabolism, photosynthesis, and sucrose phloem loading [8]. Melatonin probably achieved its promotional roles in soybean through up-regulation of genes involved in carbohydrate metabolism [9]. While the activities of sugar synthesis and metabolism-related enzymes were changed under different environment. Thus, in this study, the purpose was to investigate the changes of sugar contents as well as the related enzymes activities under excess nitrate stress with/without melatonin.

2. Material and methods

The experiments were carried out in climate-control chambers, with a relative humidity (RH) of 60-80%, temperature of 25/15°C (day/night), 16/8 h photoperiod and light intensity of 300 μmol m⁻² s⁻¹. Lettuce plants (Lactuca sativa cv. Da Su Sheng) were used as materials. Seedlings after germination for 25 days were transferred to plastic tubs for hydroponic cultivation. Half-strength Japanese Garden formula for nutrient solution was used. The nutrient solution contained deionized water at a pH of 6.0 ± 0.1 and was changed every 3 days, provided with dissolved oxygen by air pumps.

The 40-day-old seedlings, with 6 leaves in basic vegetative period, were treated as follows:①CK, control; ②M, adding melatonin; ③S, adding NO₃⁻; ④M+S, adding NO₃⁻ + melatonin. The analytical purity of Ca(NO₃)₂ with final concentrations of NO₃⁻ (160 mM) according to Yuan [1] added into nutrient solution, and melatonin were added into nutrient solution separately or combined with Ca(NO₃)₂; the concentration of melatonin were followed the results of our previous experiments (data not shown). Leaves were sampled from 10-day-stressed seedlings. The second fully expanded leaves from the top of the plants were cleaned and sampled with liquid nitrogen and stored for the rest investigation.

For the determination of sucrose, glucose and fructose, sugar content was separated and determined by high performance liquid chromatography (HPLC). Sugar-Park I (Water, part WAT085188), water Millemgium software control and data processing [10].The enzymatic activities of sucrose phosphate synthase (SPS) and sucrose synthase (SS) were determined by the method from Verma et al [11]. The activities of acid invertase (AI) were determined by the method from Miron and Schaffer [12].

Statistical analysis was carried out by using SAS statistical software. Values represent the mean ± SE standard deviation of 3 replicate samples. Letters indicate significant differences at p<0.05 according to Duncan’s multiple range tests.

3. Results and discussion

3.1 Effect of melatonin on lipid peroxidation and antioxidant enzymes activities

From the figure 1, excess nitrate stress significantly increased MDA levels compared with control, but exogenous melatonin decreased the MDA content in stressed seedlings. No changes were found between the melatonin alone and the control in MDA level. There is similar result found in H₂O₂ level, i.e., melatonin significantly reduced the H₂O₂ level compared with that of stress alone treatment. On the other hand, excess nitrate stress significantly reduced SOD activity but the exogenous melatonin reversed the decrease (Table 1). Excess nitrate stress had no effect on the activity of POD and the activity of CAT, while there was an increase in the POD induced by melatonin under the stress. According to previous studies, melatonin can alleviate oxidative damage of plants under stress by improving their antioxidant capacity [13]. Melatonin can scavenge some free radicals, but cannot directly scavenge O₂⁻[14]-[15]. Therefore, the regulation and protection of melatonin on enzyme activity can improve the ability to adapt to the stress of excessive nitrate.
Figure 1. Effects of melatonin on the content of MDA and H$_2$O$_2$ in leaves.

Table 1. Effects of melatonin on antioxidant enzyme activities in leaves.

| Treatments | SOD activity (U/mg protein) | POD activity (U/mg protein) | CAT activity (U/mg protein) |
|------------|-----------------------------|----------------------------|----------------------------|
| CK         | 22.3035±2.29515a            | 124.989±6.7802b            | 139.282±7.47146ab          |
| M          | 21.7356±2.05152a            | 117.817±8.49895b           | 143.861±8.28804a           |
| S          | 13.9005±1.29117b            | 127.879±5.13701b           | 122.041±4.15107b           |
| M+S        | 25.3706±0.3007a             | 150.804±3.16555a           | 136.406±3.90381ab          |

Values are means ± standard errors. Means with the same letter within each column are not significantly different at p < 0.05.

3.2 Effect of melatonin on contents of sucrose, glucose and fructose in leaves
Sucrose, glucose and fructose in lettuce leaves were determined in this experiment (shown in table 2). Compared with the control, excess nitrate stress increased the contents of sucrose, glucose and fructose. After 10 days of stress, sucrose, glucose and fructose in leaves were 3.4, 1.5 and 1.4 times higher than those in the control, respectively. Melatonin treatment further increased sugar content, sucrose, glucose and fructose were 3.8, 2 and 1.6 times as much as the control. Under non-stress conditions, melatonin increased the content of glucose. Maintaining the dynamic balance of sucrose synthesis, transport and metabolism is beneficial to plant adaptation to environmental changes. At low concentrations, sucrose can be used as a substrate or stress-inducing signal, while at high concentrations, it can be directly used as a protective agent [5]. And the hexose, like glucose and fructose, also are involved directly or indirectly in stress response [16]. Thus, melatonin may enhance resistance of the lettuce plants to excess nitrate stress by promotion of sugars.

Table 2. Effect of melatonin on contents of sucrose, glucose and fructose in leaves.

| Treatments | Sucrose (mg/gFW) | Glucose (mg/gFW) | Fructose (mg/gFW) |
|------------|-----------------|------------------|------------------|
| CK         | 0.94688±0.03683c| 1.38827±0.01877c| 2.60307±0.02831b|
| M          | 1.07967±0.20091c| 2.16423±0.31815b| 2.77042±0.33699b|
| S          | 3.22528±0.0066b | 2.12213±0.0533b | 3.74931±0.02322a |
| M+S        | 3.63008±0.08812a| 2.78889±0.08908a| 4.24878±0.11056a |

Values are means ± standard errors. Means with the same letter within each column are not significantly different at p < 0.05.

3.3 Effect of melatonin on the activities of enzymes involved in sucrose synthesis and metabolism in leaves.
As is shown in table 3, excess nitrate stress had significant effects on the activities of sucrose phosphate
synthase (SPS), sucrose synthase (SS-CLEAGE) and acid invertase (AI) related to sucrose synthesis and cleavage. Excess nitrate stress significantly increased the activity of SPS and SS while decreased the activity of AI. Under nitrate stress, melatonin treatment further increased SPS, which was consistent with the results in cucumber [16]. Galtier et al [17] showed that the content of sucrose, glucose and fructose could be increased by improving SPS activity of tomato through transgenic technology, which could explain the result of increase in sugars in this experiment (shown in table 2). And melatonin treatment inhibited the decrease of AI activity. Under non-stress conditions, melatonin increased the activity of AI, but had no significant effect on other sucrose metabolism-related enzymes mentioned above. Zhao et al [8] has found the application of melatonin increased expression of AI gene in barley. We need further study to explore the effect of melatonin on expression of AI under excess nitrate stress.

However, the activity of SS-CLEAGE in the present of melatonin had no difference from that of excess nitrate stress alone. Melatonin also did not increase the activity of SS-CLEAGE under non-stress conditions. Although some studies reported that melatonin could increase the expression of SS under non-stress conditions [8]. This may be related to the post-transcriptional and post-translational regulation of SS-CLEAGE [18].

Table 3. Effect of melatonin on the activities of the activities of sucrose phosphate synthase (SPS), sucrose synthase (SS-CLEAGE) and acid invertase (AI) in leaves.

| Treatments | SPS (μmol / g protein min) | SS-CLEAGE (μmol / g protein min) | AI (μmol / g protein min) |
|------------|---------------------------|----------------------------------|---------------------------|
| CK         | 28.576±0.21193c           | 57.554±4.15505b                  | 159.715±5.1924b           |
| M          | 28.8573±0.57585c          | 51.936±2.07461b                  | 190.467±4.4455a           |
| S          | 38.3629±0.81239b          | 100.251±6.9995a                  | 88.363±0.7761c            |
| M+S        | 45.6069±1.33057a          | 91.289±3.66519a                  | 154.465±2.7979b           |

Values are means ± standard errors. Means with the same letter within each column are not significantly different at p < 0.05.

4. Conclusion
Under excess nitrate stress, the increase of SPS and SS-CLEAGE and the decrease of AI may be the stress reaction. Exogenous melatonin enhanced the activities of SPS and AI enzymes under stress, which is conducive to the improvement of sucrose content and metabolic capacity, maintaining the dynamic balance of sucrose synthesis and metabolism. At the same time, there were also effective ROS scavengers by both antioxidant enzymes and sugars. Therefore, except for the effective antioxidant system, melatonin may induce the sugar-antioxidant network to enhance the plant resistance to excess nitrate stress.

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