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Full Length Article

Effects of Dicyandiamide on Physiology and Nitrate Accumulation of Hydroponic Cherry Radish

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Abstract

Nitrate is an important factor affecting the safety of vegetables. How to reduce the content of nitrate has become a common concern in vegetable production. The effect of dicyandiamide (DCD) on physiology and nitrate accumulation was studied using hydroponic cherry radish as material, in order to lay a foundation for production radish with better quality. The results showed that DCD treatment increased the content of chlorophyll-a (Chl-a), carotenoid (Car) and anthocyanin while decreased Chl-b content. Treatment with 3% DCD reduced the nitrate content while 6% DCD increased it compared with the control. Furthermore, higher the nitrate content, lower was the nitrate reductase (NR) activity, which suggested that there was a negative correlation between nitrate content and NR activity in leaves of cherry radish. The stomatal density of leaves was almost the same between 3% DCD and the control, while it was lower under 6% DCD. To sum up, DCD significantly affected the photosynthetic pigment content of cherry radish, which may affect the photosynthesis. DCD also reduced the nitrate accumulation as well as increased the Car and anthocyanin content. These findings suggested that application of DCD may be an approval measurement to improve safety and appearance quality of hydroponic cherry radish.

Keywords: Dicyandiamide; Photosynthesis; Nitrate; Cherry radish

Introduction

Nitrogen (N) is very important for the growth and development of crops (Jones *et al*. 2005). As the expanding of the population, the demand for food continues to increase. In order to improve crop yields, large amounts of nitrogen fertilizer, particularly nitrate fertilizer, are widely used (Bian *et al*. 2020). Excessive utilization of nitrogen fertilizer reduced the efficiency of nitrogen fertilizer, led to environmental contamination, accelerated soil acidification, caused the accumulation of nitrate in plants and reduced the quality of crops (Popa *et al*. 2021). Temme *et al*. (2011) thought nitrate was one of the most important factors influencing the quality of crops.

Vegetables are among the most important sources of food which provides essential vitamins, minerals and secondary metabolites for human beings. Meanwhile, vegetable is also one kind of crops that are particularly easy to accumulate nitrate. More than 80% of human nitrate comes from vegetables (Velzen *et al*. 2008). Vegetables with high nitrate content was a threat to human health, because it may lead to gastric cancer and methemoglobinemia (Song *et al*. 2015). The nitrate problem in vegetables has gradually attracted the attention of scientists and farmers (Bian *et al*. 2018). The European Union has limited the maximum nitrate content of fresh spinach to 2000 mg/kg (Irigoyen *et al*. 2006).

Nitrate is absorbed by the roots, but only a small part of it is assimilated in the roots, the rest was assimilated in the leaves or other organs by nitrate reductase (NR) and other metabolic enzymes (Lam *et al*. 1996). So, the final nitrate content in plants depends on the absorption, transportation and metabolism of nitrate. Studies showed that the absorption, assimilation and transportation of nitrate in plants were affected by both internal (plant itself) and external factors (environmental factors). The content of nitrate varied among vegetables, growth stages and plant tissues. Escobar-Gutierrez *et al*. (2002) demonstrated that nitrate content varied greatly among varieties. Nitrate content of white leaf lettuce was significantly higher than that of common variety (Reinink *et al*. 1994). The nitrate content of dark and wrinkled spinach was significantly higher than that of light and smooth spinach (Barker *et al*. 1974). Transport capacity of plants to nitrate was affected by the concentration of external nitrate. When NO_3^- was added into the culture medium, the activity of high-affinity transport system of barley was significantly increased (Aslam *et al*. 1992). The induced high-affinity transport system also had stronger nitrate induction effect (Yaeesh *et al*. 1990) and the absorption capacity of nitrate nitrogen in plants was inhibited by NH_4^+ (Aslam *et al*. 1994). How to reduce nitrate content in plants has become a concern of everyone. It was well known that nitrification was one of a cause for nitrate

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accumulation. So, nitrification inhibitors such as dicyandiamide (DCD) have been applied to regulate on nitrate accumulation in plants. Irigoyen *et al*. (2006) showed that DCD reduced the nitrate content of spinach by 18–61% under Mediterranean growing conditions. Elrys *et al*. (2021) concluded that DCD can limit nitrate content of potatoes to acceptable levels.

The radish (*Raphanus sativus* L.) belongs to the Brassicaceae family, which is a kind of vegetable that is easy to accumulate nitrate. In this study, seedlings of hydroponic cherry radish were used as the materials to study the effects of DCD on physiological changes and nitrate accumulation, hope to lay a foundation for study of the nitrate regulation in cherry radish in future.

Materials and Methods

Plant material

Seeds of cherry radish (*Raphanus sativus* L. var.radculus pers) namely Tianqian (produced by Meifa seed Co. Ltd, China) were selected and soaked with water for 4 h, then sown in the plugs (54 cm×28 cm) with mixtures of peat and vermiculite (3:1 w/w). Seedlings were cultured at 20±1ºC chamber with carefully management until grew to three leaves.

Selected healthy and uniform size seedlings with three leaves, cleaned the roots with deionized water, then cultured in the boxes filled with Hoagland nutrient solution. After two-day culture, replaced the nutrient solution with new ones containing different concentration of DCD (0, 3 and 6%). Leaves were harvested for assessment of the following indexes at the 1^{st}, 3^{rd}, 5^{th}, 7^{th} and 9^{th} day of treatment. All experiments were randomized block design with 3 replicates.

Pigment analysis

The contents of chorophyll-a (Chl-a), Chl-b, Chl-a+b, and carotenoid (Car) were determined according to the method of Wang *et al*. (2008). A 1.0 g of leaf tissue was homogenized with 80% acetone, centrifuged at 12,000×g for 8 min. The supernatants were used for determination at 662, 645 and 440 nm with a UV-5200 spectrophotometer (Shanghai Metash Instruments Co. Ltd, Shanghai, China).

Anthocyanin content assay

The content of anthocyanin was assayed with the method of Wang *et al*. (2019) with some modifications. A 1.0 g of leaf tissue was incubated in 20 mL acidified methanol (with 1% HCl) at room temperature for 12 h. The extract was use for the measurement of anthocyanin content at 530 and 600 nm with a UV-5200 spectrophotometer.

Measurement of nitrate content

Nitrate content was determined via the method of salicylic acid (SA) according to Machado *et al*. (2021) with some modifications as following steps. A 1.0 g of leaf tissue was homogenized with 15 mL ddH_2O, incubated at 90^0C for 30 min, cooled at room temperature for 30 min, centrifuged at 12,000×g for 15 min, transferred the supernatant to a 25 mL volumetric flask, and the volume was fixed to scale with ddH_2O. Took the extract (0.1 mL) to a new tube, added 0.4 mL solution of 5% SA-H_2SO_4, mixed carefully, cooled at room temperature for 20 min, added 9.5 mL NaOH (8%), cooled at room temperature again for 30 min. The OD of the reaction solution was measured at 410 nm.

Measurement of NR activity

NR activity was assayed with the method of Zhang *et al*. (2021). A 0.5 g of leaf tissue was homogenized with 5 mL buffer (100 m*M* Tris/HCl pH 8.0, 1 m*M* EDTA, 10 m*M* cysteine), then centrifuged at 12,000 g for 10 min. The supernatant was used for NR determination.

Stomatal density

Stomatal density was determined according to Kumar *et al*. (2021). A thin layer of transparent nail polish was applied on detached leaves, peeled off after being dried according to the method of Kusumi (2017), then observed stoma at 40× magnification with an Olympus imaging system.

Statistical analysis

Data were subject to analysis of variance (ANOVA) and analyzed using SPSS19.0. Differences between the means were separated by Duncan's Multiple Range Test ($P < 0.05$).

Results

Effects of DCD on pigment content in cherry radish leaves

Chl-a content: Chl-a content showed an upward trend during the period of treatment (Fig. 1). In the first three days, there was little difference in Chl-a content among three treatments, and the differences appeared from the fifth day. On the fifth day, the Chl-a content of 6% DCD was the highest, then followed by the control, and 3% DCD was the lowest. From the 7^{th} day, Chl-a content of both 3 and 6% DCD were significantly ($P < 0.05$) higher than that of the control. On the 7^{th} and 9^{th} day of treatment, Chl-a content of 3 and 6% DCD were 1.23, 1.15 and 1.35, 1.18 times higher than the control, respectively. In conclusion, DCD increased the content of Chl-a in cherry radish leaves, but this effect was related to the treatment time, that was to say the effect was shown only in the late stage of the DCD treatment.

Chl-b content: The change of Chl-b content was similar to that of Chl-a under DCD treatment. The difference was not significant ($P > 0.05$) at the early stage of treatment (1–5 d), but it became significantly ($P < 0.05$) obvious later (7–9 d)

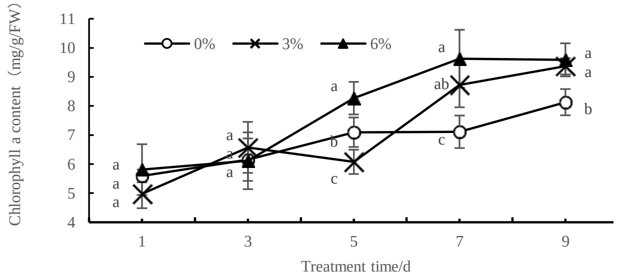

Fig. 1: Chl-a content in leaves of cherry radish
Note: In this and subsequent figures, 0% : control; 3%: 3% DCD; 6%: 6% DCD. Data are the mean ± SD of three repeats. Different letters are significantly different at P < 0.05, the same as below

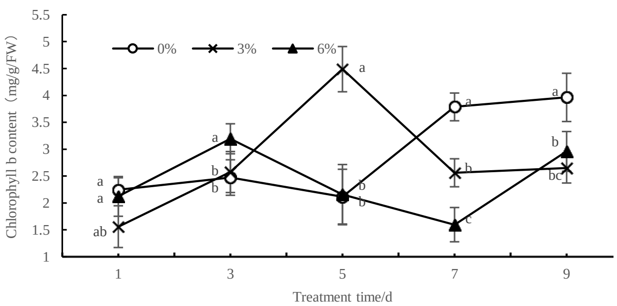

Fig. 2: Chl-b content in leaves of hydroponic cherry radish

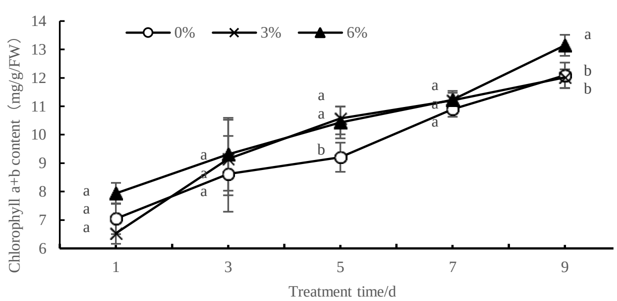

Fig. 3: Chl-a+b content in leaves of hydroponic cherry radish

(Fig. 2). From the 7th day of the treatment, the contents of Chl-b of both 3 and 6% DCD were all lower than those of the control, which indicated that DCD treatment reduced the content of Chl-b at this period.

Chl-a+b content: It can be seen that with the treatment of the time prolong, the content of Chl-a+b increased (Fig. 3). It also showed that from the 1st to 3rd day, the contents of Chl-a+b in three treatments were almost the same without any differences (Fig. 3). On the 5th and 9th day, the differences appeared, the Chl-a+b content of 6% DCD treatment was 1.13 times and 1.09 times of the control, respectively. But on the 7th day, the difference disappeared. It suggested that DCD treatment increased the content of Chl-a+b of radish only on certain periods, but this need to be studied further.

Car content: During the treatment period, the content of Car increased in all three treatments (Fig. 4). The Fig. 4 also showed that the Car content of 6% DCD was significantly

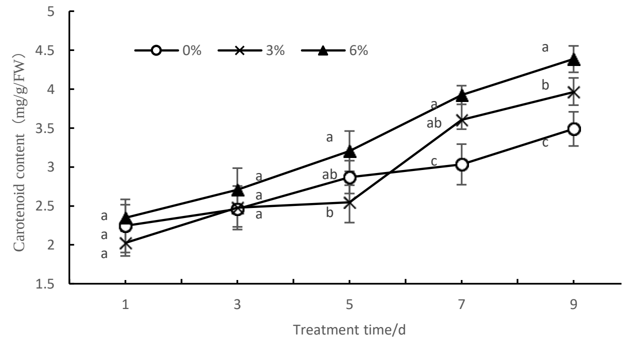

Fig. 4: Car content in leaves of hydroponic cherry radish

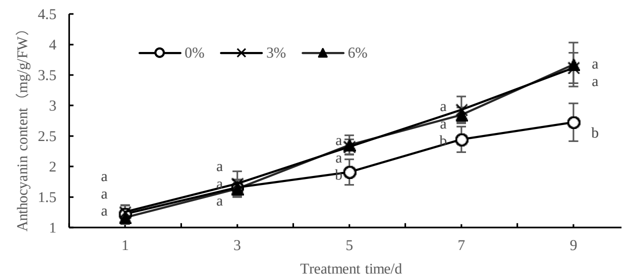

Fig. 5: Anthocyanin content in leaves of hydroponic cherry radish

higher than that of control from the 5th to 9th day and the Car content of 3% DCD was also significantly higher than that of control from the7th to 9th day (Fig. 4) ($P < 0.05$). On the 7th and 9th day, Car content of 3 and 6% DCD was 1.12, 1.29 times and 1.14, 1.26 times of the control, respectively. In conclusion, DCD treatment increased the Car content of cherry radish and the effect under 6% DCD was better.

Anthocyanin content: With the extension of treatment time, anthocyanin content increased in all three treatments (Fig. 5). From the 5th day onwards, the anthocyanin content of 3 and 6% DCD was significantly higher than that of the control (Fig. 5) ($P < 0.05$). On the 5th, 7th and 9th day, the anthocyanin content of 3% DCD was 1.21, 1.20, 1.33 times of the control (0%), respectively. For the 6% DCD group, it was 1.23, 1.17, 1.34 times of the control, respectively. However, there was little difference between 3 and 6% DCD treatment. It concluded that DCD promoted anthocyanin accumulation in cherry radish leaves.

Effects of DCD on nitrate accumulation in leaves

During the whole treatment time, the nitrate content of leaves in all treatments increased (Fig. 6). Among the three treatments, the nitrate content of 3% DCD was the lowest. There was no significant difference in nitrate content between 3% DCD and the control from the first day to the fifth day ($P > 0.05$). From the 7th day to 9th day, nitrate content of 3% DCD was significantly lower than the control ($P < 0.05$). Therefore, 3% DCD treatment inhibited the accumulation of nitrate in leaves. There was no significant difference in nitrate

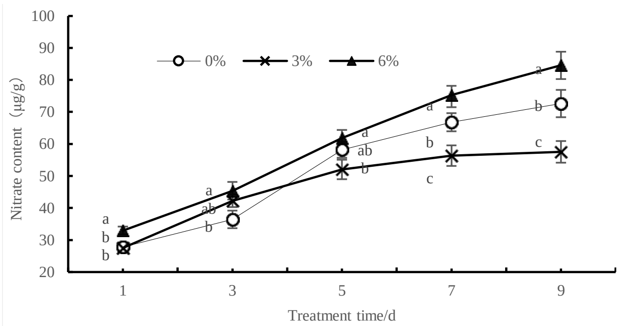

Fig. 6: Nitrate content in leaves of hydroponic cherry radish

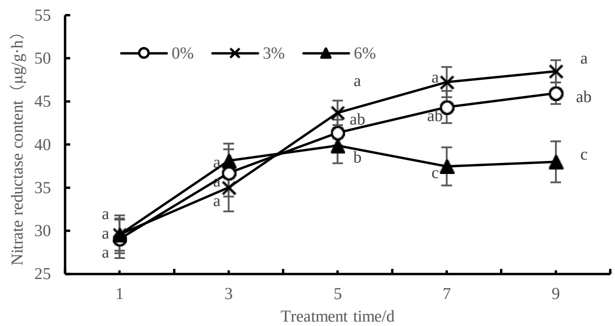

Fig. 7: NR activity in leaves of hydroponic cherry radish

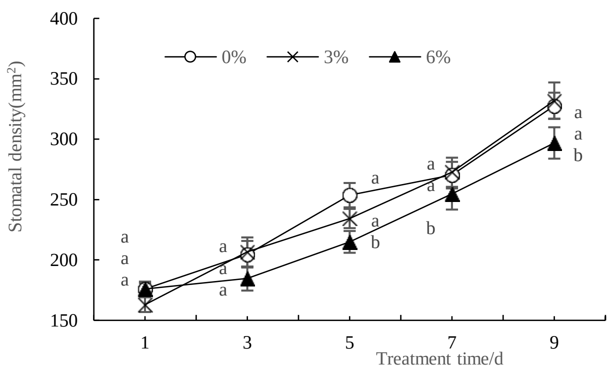

Fig. 8: Stomatal density in leaves of hydroponic cherry radish

content between 6% DCD and the control from the first day to the fifth day (P >0.05). But on the 7th day, the nitrate content of 6% DCD was significantly higher than that of the control (P <0.05). It can also be seen from Fig. 6 that the curve of the 6% DCD was always above the control, which suggested that this treatment promoted nitrate accumulation in leaves of cherry radish.

On the 5th, 7th and 9th day of the treatment, the nitrate content of 3% DCD was 0.89, 0.84 and 0.79 times, while the 6% DCD was 1.06, 1.14, 1.16 times of the control, respectively. In conclusion, 3% DCD has an inhibitory effect on nitrate accumulation in cherry radish leaves, while 6% DCD did an opposite effect.

DCD treatment also affected NR activity of radish leaves (Fig. 7). During the period of treatment, NR activity showed an upward trend, which was similar to the change of nitrate content (Fig. 6). Compared with the control, the NR activity of 3% DCD showed no significant difference in the first three days (P >0.05), but it was higher than that of the control from the 5th to 9th day. There was no significant difference in the NR activity between 6% DCD treatment and the control in the first 5 days (P >0.05). But from the 7th to 9th day, NR activity of 6% DCD was lower than the control. On the 5th, 7th and 9th day of treatment, the NR activity of 3% DCD was 1.05, 1.07, 1.06 times of the control, while it was 0.96, 0.85, 0.83 times of the control under 6% DCD, respectively. In conclusion, 3% DCD treatment increased NR activity in cherry radish leaves, but 6% DCD treatment inhibited it, which suggested that the influence of DCD on NR activity may be related to DCD concentrations. The results further showed that there was a negative correlation between nitrate content and NR activity in cherry radish leaves under DCD treatment.

Effects of DCD on stomatal density in leaves of cherry radish

Stomatal density in all treatments increased all the time (Fig. 8). Difference was not significant between the 3% DCD treatment and the control (P>0.05). However, 6% DCD influenced stomatal density obviously. From the 5th to 9th day, the stomatal density in 6% DCD treatment was significantly lower than both control and 3% DCD (P<0.05). These results indicated that 6% DCD treatment had a certain inhibitory effect on the increase of stomatal density, while 3% DCD did not.

Discussion

Fertilizer is very important for crops. However, unreasonable use of fertilizers can cause nitrate accumulations in plants, which was harmful to human beings (Popa *et al.* 2021). Reducing nitrate content in vegetables has become a concern of everyone, and various measures are being explored. Management of the nitrogen fertilizer was a common measure for reducing the nitrate accumulation. Liu and Yang (2012) verified that reducing nitrate concentration in nutrient solution significantly reduced nitrate content of lettuce. However, reducing inputs of nitrogen fertilizer also affected crop growth and development, thus led to yield loss. Application of nitrifying inhibitor such as DCD to regulate nitrate was seemed to be a suitable measure. Yu *et al.* (2006) showed that adding 10–20% DCD reduced the nitrate content in leaves and stems of radish by 29.9–34.6% and 11.7–32.2%, respectively. The results of this experiment agreed with the above. Since NR is one of important enzymes in the process of nitrogen metabolism, it is also a rate limiting enzyme in the process of nitrogen uptake, assimilation and translocation. The activity of NR determines the rate of nitrate assimilation into organic nitrogen compounds; therefore, the decrease of nitrate accumulation in plants by DCD may be related to NR activity.

It was thought that a higher NR activity promoted the reduction of nitrate, thus reduced nitrate accumulation levels. For example, low nitrate content of pakchoi, its amount of NR activity and gene expression was higher (Luo *et al.* 2006). The nitrate content of potato transformed by exogenous NR gene was also significantly reduced (Djennane *et al.* 2002). It seemed that NR activity was negatively correlated with nitrate content, which was similar to the results of this experiment. However, the results of Blom-Zandstra and Alberr (1986) suggested that nitrate content was positively correlated with NR activity. Analysis showed that the relationship between nitrate content and NR activity may be associated with many factors, such as plant tissue, culture method, growth environment, etc. Nitrate is absorbed by roots, most of it is transported to other tissue and organs including leaves to assimilate under NR and other enzymes, which may be affected by many factors. Liu *et al.* (2006) believed that the influence on nitrate content might not be the absolute value of NR, but the expression degree of potential NR activity. The higher the potential NR activity, the more nitrate is reduced and less accumulated in plant tissue (Liu *et al*. 2006). Nitrate content may also be related to the ratio of endogenous/exogenous NR activity (Huang *et al.* 2012).

Chl-a and Chl-b were similar in structure except that the side chain at C-7 was a methyl group in Chl-a, while it was a formaldehyde group in Chl-b. Biosynthesis of Chl-b started and ended with Chl-a, which was considered to be a "Chl cycle". Biosynthesis of Chl-a need only one step while Chl-b need two steps. Plants adjusted the Chl-a/b ratio according to different conditions (Wang *et al.* 2008). Many studies found that as long as the Chl-b content decreased, there must be an increase in Chl-a content (Akoyunoglou and Akoyunoglou 1985; Tanaka *et al.* 1991). The results of this study was consistent with the results above, which suggested that DCD affected the Chl metabolism of cherry radish. Perhaps, there were some action sites for DCD in the Chl metabolism. Anyway, future research needs to be identified and studied.

Conclusion

DCD increased the content of Chl-a, Car and anthocyanin of cherry radish. 3% DCD also deduced the nitrate accumulation. Data indicated that DCD not only affected the photosynthesis, but also improved the color and reduced nitrate accumulation of cherry radish. It is well-known that Chl is an important pigment for photosynthesis, Car and anthocyanin are important color pigments and nitrate is harmful for human beings. So, application of a certain amount of DCD in cherry radish production may be an approval method to improve its appearance and safety quality.

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Author Contributions

WF planned the experiments, LG and ZX interpreted the results. WF and LG made the write up and JY statistically analyzed the data and made illustrations.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper.

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