Grafting Improves Growth and Nitrogen-use Efficiency by Enhancing NO₃⁻ Uptake, Photosynthesis, and Gene Expression of Nitrate Transporters and Nitrogen Metabolizing Enzymes in Watermelon Under Low Nitrogen

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Research Article

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Abstract

Background Excessive and insufficient application of N fertilizer can inhibit plant growth, reduce N-use efficiency (NUE) and lead to production reduction. Watermelon is an important crop that often restricted by inappropriate N supply. The study aims to test whether grafting with bottle gourd rootstock can improve NUE and growth performance of watermelon under low N and to clarify the underlying physiological mechanism.

Methods Grafted (self-grafted and rootstock-grafted watermelon) and ungrafted (watermelon and bottle gourd) seedlings were tested respectively, and treated with 9 mM (normal condition) and 4 mM (low N condition) NO_3^- -N concentrations for 18 days under hydroponic conditions.

Results The growth and NUE of bottle gourd rootstock-grafted watermelon seedlings increased under low-nitrate, while decreased slightly in self-grafted seedlings compared with control. Rootstock-grafted plants had higher root morphological characteristics, NO_3^- uptake, photosynthesis, and NUE traits than self-grafted plants under low-nitrate. The expression of nitrate transporter genes *NRT1.5* and *NRT2.1*, and N metabolizing enzyme genes *NR*, *NiR*, *GS1* and *GS2* of rootstock-grafted plants were significantly up-regulated at low-nitrate treatment, which may lead to increased NO_3^- uptake and metabolism.

Conclusion The bottle gourd rootstock grafting can improve the growth and NUE of watermelon seedlings under low-nitrate treatment. The improved plant performance is attributed to the vigorous root systems and higher NO_3^- uptake of rootstock roots, and the enhanced N metabolism and photosynthetic capacity of scion leaves. Grafting with the bottle gourd rootstock may be beneficial to the efficient production of watermelon and economic application of N fertilizer.

Introduction

Nitrogen (N) is one of the most important mineral nutrients required by crops, and is a key limiting factor for plant growth and productivity (Iqbal et al. 2019). Many important organic compounds in plants, such as protein, nucleic acid, chlorophyll, enzyme, vitamin and alkaloid, are inseparable from the participation of nitrogen, which plays an important role in plant life activities. In China's greenhouse crop production, large-scale application of N-fertilizer has become the main means to improve crop productivity. However, blindly applying a large amount of N-fertilizer will not only reduce crop N-use efficiency (NUE), increase agricultural production investment, cause serious fertilizer waste, but also lead to a series of environmental problems such as soil secondary salinization (Yuan et al. 2014). Thus, improving NUE and reducing N fertilizer input without decreasing crop growth and yield are imperative and great current challenges for sustainable development of agriculture.

Nitrate nitrogen (NO_3^- -N) is the nitrogen with the highest bioavailability and most absorbed by plants (Zhen et al. 2018). NO_3^- uptake by cells is mediated by nitrate transporter proteins (NRTs) located on the plasma membrane of plant roots. This process is completed by high affinity transport systems (HATS) and low affinity transport systems (LATS). HATS and LATS act when the concentration of NO_3^- -N is low (<1.0 mM) and higher than 1.0 mM, respectively. These two ways work together to affect NUE by affecting N transformation. At the molecular level, the genes encoding these transporters play a crucial role in NO_3^- uptake and transportation from soil to plants (Li et al. 2018), and are divided into two gene families: NRT1 (low affinity nitrate transporter genes) and NRT2 (high affinity transporter genes) (Bucher et al. 2014). NO_3^- absorbed into the plant is reduced to nitrite under the action of nitrate reductase (NR) in the cytoplasm, and nitrite reductase (NiR) further reduces it to ammonium. High concentrations of ammonium are toxic and thus need to be rapidly assimilated by glutamine synthetase (GS) and glutamate synthase (GOGAT) to produce non-toxic organic compounds, such as glutamine and glutamate (Krapp 2015). From this, it is highly likely to promote plant growth and NUE by increasing N uptake and assimilation (Qiao et al. 2019).

Root system is an important organ for plants to absorb inorganic N (Gomes et al. 2021); therefore, its morphological structure and physiological characteristics will affect the ability of plants to obtain N (Jiang et al. 2017), which will directly affect the growth and development of plant shoots. Some studies have reported that grafting can effectively improve the nutrient

absorption of plant roots by "changing roots" (Colla et al. 2011; Özmen et al. 2015; Sallaku et al. 2019). Many suitable rootstocks have been selected for specific scion varieties and used to improve nutrient absorption and utilization, so as to promote plant growth and development (Albacete et al. 2015). Our previous study also demonstrated that the growth and NUE of cucumber seedlings can be promoted by grafting onto N-efficient rootstocks (Liang et al. 2021). Hence, grafting with suitable rootstocks is a very effective and feasible method to improve plant N uptake and the NUE.

Grafting is a widely used biotechnological tool in vegetable crops and is considered to be a rapid and effective method to protect plants against the effects of soil-borne pathogens (Rahman et al. 2021), and enhance plant's resistance to various abiotic stresses, such as high temperature (De Oliveira et al. 2020), drought (Luo et al. 2020), salinity (Lo'ay and Abo E-Ezz 2021). In addition, rootstock grafting can also improve the absorption of potassium (K) and magnesium (Mg) by plants (Huang et al. 2013, 2016), and rootstock is the decisive factor for grafted plants to absorb mineral nutrients (Huang et al. 2016; Nawaz et al. 2017; Zhen et al. 2010). Thus, it is valuable to discuss the genotype variation of rootstock effect for understanding the grafting efficacy (particularly in nutrient uptake and transport).

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nakai.] is one of the most important Cucurbitaceous vegetables and the most popular fruit in summer. As its pulp is rich in many nutrients beneficial to human health, such as water, protein, sugar, minerals, vitamin C and various amino acids. Watermelon growth requires a large amount of fertilizer, so fertilization is an important means to improve its yield. However, in recent years, the blind application of a large amount of N-fertilizer in protected watermelon cultivation in China has seriously limited the plant growth and reduced the NUE and yield of watermelon. Although several reports suggest that rootstock grafting can alleviate growth inhibition caused by abiotic stresses through improving plant N uptake and utilization (Yang et al. 2013; Özmen et al. 2015; Sallaku et al. 2019), little information is reported about gene expression and related underlying mechanisms, particularly under low N condition. Moreover, rootstocks with high N acquisition capacity may enable grafted-plants grow better with less N-fertilizer. However, this possibility needs to be further confirmed. Therefore, the purpose of this study is to investigate 1) whether bottle gourd rootstock grafting can improve the growth and NUE of watermelon seedlings under low N conditions and 2) whether the enhanced watermelon performance is attributed to higher root NO_3^- uptake and the improvement of photosynthesis and N metabolism capacity.

Materials And Methods

Plant materials and experimental treatments

Experiment 1 response of grafted watermelon to low-nitrate

The experiment was performed from September to November 2020 in a research greenhouse of Northwest Agricultural and Forestry University in Northwest China (34° 16' N, 108° 4' E). Seeds of rootstocks 'Zaojia 8424' [*Citrullus lanatus* (Thunb.) Matsum. and Nakai., Xinjiang Academy of Agricultural Sciences, China] and 'Jingxinzheng No.1' [*Lagenaria siceraria* Standl., Beijing Academy of Agricultural Sciences, China] were sown in 50-cell plug trays filled with a 2:1(v/v) mixture of peat and perlite; after 4 days, seeds of scion 'Zaojia 8424' were sown. When the seedlings of rootstock had developed one true leaf, scion was grafted onto the two rootstocks, using the "insertion grafting" procedure as described by Hassell et al. (2008). In order to maintain a relatively high temperature and humidity environment, seedlings were moved to a small arch shed covered with a layer of transparent plastic film and completely shaded for 72 hours. The plastic film was removed for a short time during initial days to control relative humidity, and it was completely removed after 7 days of grafting. The plants were irrigated with tap water before grafting and transplanting to hydroponic cultivation, and no nutrient solution was used during this period. The self-grafted 'Zaojia 8424' plants were used as control. When the two true leaves emerged, the grafted plants were transplanted into 30 L plastic containers (85 cm × 40 cm × 20 cm), each containing twelve plants. The plants were directly exposed to two levels of NO_3^- -N concentration [9 mM (control) and 4 mM (low N)]. These N levels were decided based on watermelon seedlings growth in our preliminary experiments. The hydroponic nutrient solution refers to the formula of Nawaz et al. (2017). The control nutrient solution was composed of 3.5 mM $\text{Ca}(\text{NO}_3)_2$, 6 mM KCl, 0.5 mM $\text{Ca}(\text{H}_2\text{PO}_4)_2$, 1 mM MgSO_4 , 1 mM $\text{Mg}(\text{NO}_3)_2$, 89.7 μM $\text{Na}_2\text{Fe-EDTA}$, 46.3 μM H_3BO_3 , 9.5 μM MnSO_4 , 0.8 μM ZnSO_4 , 0.3 μM CuSO_4 , and 0.1 μM

(NH₄)₂MoO₄. For low N nutrient solution, 2 mM Ca(NO₃)₂ was used, without Mg(NO₃)₂, whereas the other nutrients were the same as in the control nutrient solution.

In this experiment, four treatments were composed of two grafting combinations and two N application levels. All treatments were replicated three times with 12 plants in each replicate, and were conducted using a randomized complete block design. The nutrient solution was regularly renewed every 5 days to avoid the deficiency of any specific ions and the pH of the solution was maintained at 6.0 ± 0.5. The nutrient solution was aerated with air pumps at 2-hour intervals for 1 hour each time. The temperature of the greenhouse varied from 16 to 30°C, and the relative humidity varied from 50 to 85%. The plants were harvested at 18 days following N application.

Experiment 2 response of ungrafted watermelon and bottle gourd to low-nitrate

In this experiment, ungrafted watermelon (Zaojia 8424) and bottle gourd (Jingxinzhen No.1) were used as materials. Seeds of both were sown in 50-cell plug trays filled with 2:1(v/v) mixture of peat and perlite. The seedlings were irrigated with tap water before transplanting. When the two true leaves emerged, the uniform seedlings were transplanted into 30 L plastic containers (85 cm × 40 cm × 20 cm), and each containing 12 plants. Then the plants were directly exposed to 9 mM and 4 mM NO₃⁻-N treatments. The composition and management of nutrient solution were the same as those in Experiment 1. Each treatment was replicated three times with 12 plants in each replication. All treatments were arranged in a randomized complete block design. Plant samples were harvested for measurement of plant growth, root morphology and NO₃⁻ content at day 18 of low N treatment.

Measurement of plant growth

Five randomly selected plants per treatment were collected to measure plant height (from stem base to growing point) and stem diameter (at 2 cm above the graft union). The grafted plants were divided into roots, stems and leaves. For ungrafted plants, the part above the cotyledon node was regarded as the “shoot”, and the part below was the “root”. These organs were rinsed with deionized water, blotted carefully with tissue paper, weighed for fresh weight and then placed in an oven at 105°C for 1 hour followed by 80°C for 48 hours to determine their dry weight. The sound seedling index and root to shoot (R/S) ratio were subsequently calculated as follow:

sound seedling index = (stem diameter / plant height + root dry weight / shoot dry weight) × whole dry weight

R/S ratio = root dry weight / shoot dry weight

Analysis of root morphology

Roots of five uniform plants from each treatment were randomly selected and washed with deionized water, then placed in a dedicated tray. The entangled roots were gently separated by hand and stretched flat. The root scanning was performed by using Imagery Scan Screen (Epson perfection V700 Photo, Indonesia). Total root length, root surface area, root diameter, root volume, root tips, and root forks were measured using image analysis through WinRHIZO Pro 2012a software.

Measurement of NO₃⁻ content and NO₃⁻ uptake

The NO₃⁻ content was measured by the colorimetric method utilizing salicylic acid according to Wang et al. (2018) and was calculated from the standard curve. Then NO₃⁻ uptake was calculated as the product of NO₃⁻ content and fresh weight.

Measurement of NUE traits

The N content was measured using the Kjeldahl method described by Bremner (1965). N accumulation (NA) was obtained as the product of N content and plant dry weight (mg N) (Lawlor 2002). N uptake efficiency (NUpE), N utilization efficiency (NuTE)

and NUE of plants were calculated based on the the following formulas (Siddiqi and Glass 1981; Elliot and Laüchli 1985):

$$\text{NU}_\text{pE} = \text{N accumulation} / \text{Root dry weight (mg N / mg DW)}$$
$$\text{NU}_\text{tE} = \text{Total plant dry weight} / \text{N contents (mg DW/ mg N)}$$
$$\text{NUE} = \text{NU}_\text{pE} \times \text{NU}_\text{tE} \text{ (mg N / mg N)}$$

Determination of leaf photosynthesis

The first fully expanded true leaf from top was selected. The chlorophyll content was determined by 80% acetone extraction method according to Hussain et al. (2019). The net photosynthetic rate (Pn) and transpiration rate (Tr) were measured with a 6800 photosynthesizer apparatus from 9:00 am to 11:00 am on a sunny day.

Total RNA extraction and gene expression analysis

The root tips and leaves of grafted plants were selected to determine the relative expression of genes related to nitrate transporters (NRTs) and N metabolizing enzymes, respectively. Total RNA was extracted by using the Omega kit (Norcross, Georgia, USA) according to the manufacturer's instructions and treated with RNase-free DNase to remove contaminated DNA. First-strand cDNA was synthesized using M-MLV reverse transcriptase, and oligo-(dT)₁₈ was used as a primer following the manufacturer's recommendation [Accurate Biotechnology (Hunan) Co.,Ltd, Changsha, China]. Expression of the target genes was measured by quantitative real-time PCR (qRT-PCR). The watermelon NRT genes (Cla008783, Cla005079) and N metabolic genes (NR: Cla023145, NiR: Cla13062, GS1: Cla015195, GS2: Cla021675), and bottle gourd NRT genes (Lsi05G000610, Lsi03G001360) were selected as target genes. These genes were taken from the Cucurbitaceae Genome database (<http://www.icugi.org/cgi-bin/ICuGI/genome/search.cgi>). The specific primers (Table 1) were designed as described by Kong et al. (2014) using the Primer 5 Software. All the primers show high specificity for each gene. qRT-PCR was performed using the PerfectStart Green qPCR SuperMix kit (TransGen Biotech, Beijing, China) according to the protocols. PCR amplification included a min preincubation step at 94°C, followed by 40 cycles of 94°C for 5 s, 60°C for 30 s. The PCR products were quantified by the ABI Step One Plus real time PCR detection system and the data were analyzed by using $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen. 2001). Self-grafted and rootstock-grafted watermelon seedlings under 9 mM N treatment were used as control to calculate the relative gene expression.

Table 1
List of primer sequences of watermelon and bottle gourd used for qRT-PCR analysis

Gene	Accession Number	Forward primer(5'-3')	Reverse primer(5'-3')
Watermelon			
NRT1.5	Cla008783	TGTTGGTGACAATGGTGATG	ATCACAGTCAAGGCAGCAAG
NRT2.1	Cla005079	CAGATCATTGGGCTTGTGA	AAGGGAACCAGGGATAAAG
NR	Cla023145	GCACCGACAGCATTCTCA	CGGTATTCCTCCAGCATT
NiR	Cla13062	CGATCTATACGAACACCCTC	CCAGCACATTGAACCCTA
GS1	Cla015195	AGAGCACATTGCTGCCTATG	AGGCTCCTCTGTTTGCAACT
GS2	Cla021675	GGAGCTTATCCAGGTCTCTCA	AAGCCTTGTAGTGGGCATCT
CIAct*	Cla007792	CCATGTATGTTGCCATCCAG	GGATAGCATGGGGTAGAGCA
Bottle gourd			
NRT1.5	Lsi05G000610	TCAATGAACCGTGGTAGAAA	ATGGTGCAGAGCCAAATAG
NRT2.1	Lsi03G001360	TAATTGGGCTTGTGAATGG	AAGGGAACCAGGGATAAAA
LsiAct*	Lsi02G025660	GGCAGTGTTGTGAACATGT	CCCATGCTATCCTCCGTCTT
* is reference gene (ACT) for qRT-PCR analysis			

Statistical analysis

A two-factorial ANOVA was performed to examine the effects of graft combination, N treatment, and their interaction on the plant samples. Significance levels were determined at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; ns denoted non-significant differences. Tukey HSD ($p < 0.05$) was used for the mean separation. Pearson's correlation analysis was used to analyze the correlation between NUE and other parameters of the self-grafted and rootstock-grafted watermelon seedlings under 9 mM and 4 mM NO_3^- -N conditions, and the graphical presentation was carried out using OriginPro 2021. All analyses were conducted using SPSS 24.0 software package.

Results

Plant growth

For grafted plants, graft combination significantly affected the plant growth, and its interaction with N treatment significantly affected the plant height, shoot and whole dry weight (Table 2). The growth performance of bottle gourd rootstock-grafted (Z/J) plants were better than that of self-grafted (Z/Z) plants (Fig. 1a), correspondingly, the plant height, shoot, root, and whole dry weight, and sound seedling index of Z/J plants were all significantly higher than those of Z/Z plants regardless of the N level (Table 2). Compared with 9 mM N treatment, these growth parameters of Z/Z plants decreased slightly under 4 mM N treatment, while increased in Z/J plants. For example, 4 mM N treatment reduced plant height, root, shoot, and whole dry weight, and sound seedling index of Z/Z plants by 22%, 14%, 27%, 25% and 17%, respectively, whereas increased the plant height, shoot and whole dry weight, and sound seedling index of Z/J plants by 22%, 28%, 23% and 2%, respectively.

Table 2
Effect of graft combination and N treatment on plant growth of the watermelon seedlings

Graft combination	N treatment	Plant height (cm)		Root dry weight (g)		Shoot dry weight (g)		Whole plant dry weight (g)		Sound seedling index	
Z/Z	9 mM	26.0 ± 0.36c	100%	0.14 ± 0.01b	100%	0.71 ± 0.05c	100%	0.85 ± 0.06c	100%	0.30 ± 0.03b	100%
	4 mM	20.4 ± 0.35d	78%	0.12 ± 0.01b	86%	0.52 ± 0.03c	73%	0.64 ± 0.04c	75%	0.25 ± 0.03b	83%
Z/J	9 mM	38.8 ± 1.09b	100%	0.35 ± 0.02a	100%	1.38 ± 0.10b	100%	1.73 ± 0.12b	100%	0.60 ± 0.03a	100%
	4 mM	47.2 ± 0.50a	122%	0.34 ± 0.03a	97%	1.76 ± 0.09a	128%	2.12 ± 0.11a	123%	0.61 ± 0.04a	102%
Analysis of variance											
N treatment (N)		*		ns		ns		ns		ns	
Graft combination (G)		***		***		***		***		***	
N×G		***		ns		**		**		ns	
Data in the columns are the mean ± standard error (n=5). Different letters indicate significant differences determined by Tukey test at P≤0.05. Results of a two-way ANOVA are indicated, *P≤0.05; ** P≤0.01; *** P≤0.001; ns, not significant. Z/Z: self-grafted watermelon seedlings; Z/J: rootstock-grafted watermelon seedlings											

For ungrafted plants, bottle gourd (J) plants showed better growth performance than watermelon (Z) plants under 9 mM and 4 mM N levels (Fig. 2a). Rootstock genotype significantly affected the plant growth (Fig. 3). The root and shoot dry weights, sound seedling index, and R/S ratio of J plants were about 2.0, 1.4, 3.1 and 1.4 times of Z plants under 9 mM N treatment, respectively, and 2.2, 1.6, 3.7, and 1.4 times under 4 mM N treatment, respectively (Fig. 3).

Root morphology

For grafted plants, the root morphology traits were significantly affected by graft combination (Table 3). Between the two graft combinations, the root system of Z/J plants was larger than that of Z/Z plants (Fig. 1b), consistently, the total root length, root surface area, root volume, and root forks of Z/J plants were all significantly higher than those of Z/Z plants regardless of the N level (Table 3). Compared with 9 mM N treatment, these parameters of Z/Z plants decreased slightly under 4 mM N treatment, but increased in Z/J plants. For example, 4 mM N treatment reduced the total root length, root surface area, root volume, and root forks of Z/Z plants by 11%, 15%, 16%, and 22%, respectively, whereas increased the total root length and root forks of Z/J plants by 4% and 11%, respectively.

Table 3
Effect of graft combination and N treatment on root morphology of the watermelon seedlings

Effect of graft combination and N treatment on root morphology of the watermelon seedlings									
Graft combination	N treatment	Total root length		Root surface area		Root volume		Root forks	
		(cm)		(cm ²)		(cm ³)			
Z/Z	9 mM	491 ± 32.0bc	100%	133 ± 6.97b	100%	2.79 ± 0.20b	100%	3471 ± 396ab	100%
	4 mM	438 ± 41.2c	89%	113 ± 12.0b	85%	2.33± 0.32b	84%	2699 ± 470b	78%
Z/J	9 mM	680 ± 53.7ab	100%	266 ± 16.7a	100%	8.40 ± 0.74a	100%	3930 ± 213ab	100%
	4 mM	704 ± 58.7a	104%	267 ± 9.60a	100%	8.30 ± 0.86a	99%	4371 ± 232a	111%
Analysis of variance									
N treatment (N)		ns		ns		ns		ns	
Graft combination (G)		***		***		***		***	
N×G		ns		ns		ns		ns	
Data in the columns are the mean ± standard error (n=5). Different letters indicate significant differences determined by Tukey test at P≤0.05. Results of a two-way ANOVA are indicated, *P≤0.05,** P≤0.01; *** P≤0.001; ns, not significant. Z/Z: self-grafted watermelon seedlings; Z/J: rootstock-grafted watermelon seedlings									

For ungrafted plants, N treatment affected the total root length, while rootstock genotype and their interaction significantly affected all the root morphology traits (except that the interaction did not affect the root volume) (Fig. 4). The root system of J plants was significantly stronger than that of Z plants under 9 mM and 4 mM N levels (Fig. 2b). The total root length, root surface area, root volume, and root forks of J plants were about 1.5, 1.7, 2.0, and 2.0 times of Z plants under 9 mM N treatment, respectively, and 1.9, 2.1, 2.4, and 2.5 times under 4 mM N treatment, respectively (Fig. 4).

NO₃⁻ content and NO₃⁻ uptake

For grafted plants, the total NO₃⁻ content was significantly affected by graft combination and N treatment (Fig. 5a), and the whole plant NO₃⁻ uptake was significantly affected by graft combination and its interaction with N treatment (Fig. 5b). The total NO₃⁻ content and NO₃⁻ uptake of Z/J plants were all significantly higher than those of Z/Z plants, especially in the roots. Compared with 9 mM N treatment, 4 mM N treatment reduced the total NO₃⁻ content and NO₃⁻ uptake of Z/Z plants by 17% and 40%, while the total NO₃⁻ content of Z/J plants decreased by 9% and NO₃⁻ uptake increased by 14%. Furthermore, the NO₃⁻ content and NO₃⁻ uptake in Z/J plant roots decreased, but these values in leaves increased under 4 mM N treatment.

For ungrafted plants, both total NO₃⁻ content and NO₃⁻ uptake were significantly affected by N treatment and rootstock genotype (Fig. 6). Compared with 9 mM N treatment, 4 mM N treatment reduced the NO₃⁻ content in Z plant roots and leaves by 32% and 25%, and NO₃⁻ uptake by 38% and 42%, respectively. However, 4 mM N treatment had no significant effect on NO₃⁻ content and NO₃⁻ uptake in J plant roots, while NO₃⁻ uptake in leaves decreased by 32% (Fig. 6). Furthermore, Z plants showed significantly higher NO₃⁻ uptake than that of J plants regardless of the N level (Fig. 6b). For example, the whole plant NO₃⁻ uptake of J plants was about 1.4 times and 2.4 times higher than that of Z plants under 9 mM and 4 mM N treatment, respectively.

Gene expression of nitrate transporters and N metabolizing enzymes

Relative expression of nitrate transporters and N metabolizing enzymes genes was conducted in self-grafted and rootstock-grafted watermelon plants. According to the Cucurbitaceae Genome database, we identified two nitrate transport genes in watermelon and bottle gourd roots, respectively, and four N metabolizing enzymes genes in watermelon leaves (Table 1). The relative expression of these genes was significantly affected by N treatment, graft combination and their interaction (Fig. 7). The mRNA levels of *NRT1.5* and *NRT2.1* in the roots, and *NR*, *NiR*, *GS1* and *GS2* genes in the leaves of Z/J plants were up-regulated significantly under 4 mM N treatment compared to the 9 mM N treatment. However, these genes in Z/Z plants did not change significantly under 4 mM N treatment, except for the up-regulated expression of *NiR* and *GS2* genes. Moreover, the gene expression of nitrate transporters and N metabolizing enzymes in rootstock-grafted watermelon seedlings was significantly higher than that in self-grafted plants. For example, under 4 mM N treatment, the relative expression of *NRT1.5*, *NRT2.1*, *NiR*, *GS1* and *GS2* genes in Z/J plants were 27.3, 2.9, 2.6, 1.8 and 2.5 times higher than that in Z/Z plants, respectively.

NA, NUpE, NUtE and NUE

The NA, NUpE, NUtE and NUE were all significantly affected by N treatment, graft combination and their interaction (except that the N treatment did not affect NUtE) (Figs. 8 and 9). Obviously, the NA in roots, stems and leaves of Z/J plants was remarkably higher than that of Z/Z plants, and increased significantly under 4 mM N treatment, especially in leaves (Fig. 8). In addition, compared with 9 mM N treatment, the NUpE, NUtE and NUE of Z/J plants were increased significantly under 4 mM N treatment (Fig. 9). These NUE traits were all significantly higher than those of Z/Z plants, especially at 4 mM N level. For example, the total NA, NUpE, NUtE and NUE of Z/J plants under 4 mM N treatment were 3.8, 1.2, 3.3, and 3.8 times higher than that of Z/Z plants, respectively.

leaf photosynthesis

Graft combination significantly affected Pn, Tr and chlorophyll content, and its interaction with N treatment significantly affected the Pn and chlorophyll content (Fig. 10). The Pn, Tr and chlorophyll content of Z/J plants were all higher than those of Z/Z plants regardless of the N level. Compared with 9 mM N treatment, these parameters of Z/Z plants decreased slightly under 4 mM N treatment but increased in Z/J plants. For example, 4 mM N treatment reduced the Pn, Tr and chlorophyll content of Z/Z plants by 60%, 11%, and 16%, respectively, whereas increased by 35%, 15% and 27% in Z/J plants, respectively.

Correlation analysis

Pearson's correlation analysis between the NUE and other parameters of the self-grafted and rootstock-grafted watermelon seedlings under 9 mM and 4 mM NO_3^- -N conditions was conducted (Fig. 11). The NUE was found to be positively correlated with the plant height, shoot dry weight, whole plant dry weight, NA, NUtE, Pn, Tr, and chlorophyll content; and all correlations between these parameters above were positive. NUtE was also positively correlated with the root dry weight, sound seedling index, total root length, root surface area, root volume, and whole plant NO_3^- uptake; meantime, all these parameters were positively correlated with each other, and they were also positively correlated with the plant height, shoot dry weight, whole plant dry weight, NA and Tr (except that RV has no correlation with PH). In addition, the whole plant NO_3^- uptake was also positively correlated with Pn and chlorophyll content. The NUpE was positively correlated with the relative expression of *NRT1.5*, *NRT2.1* and *GS1* genes, and the relative expression of *NRT1.5* and *NRT2.1* genes, as well as the *NiR*, *GS1* and *GS2* genes were all positively correlated with each other.

Discussion

Bottle gourd rootstock grafting increases watermelon growth performance under low-nitrate condition

Appropriate rootstock grafting can promote plant growth and improve plant tolerance to nutrient deficiency (Huang et al. 2013, 2016). In this study, the growth performance of bottle gourd rootstock-grafted watermelon seedlings was obviously better than that of self-grafted seedlings, especially under low-nitrate condition (Fig. 1a, Table 2). The analysis of variance showed that

graft combination significantly affected plant growth, and the growth performance of ungrafted bottle gourd seedlings was also significantly better than that of ungrafted watermelon seedlings, regardless of the N level (Table 2, Figs. 2a and 3). These results demonstrated that bottle gourd rootstock grafting is an effective approach to improve watermelon growth performance under low N, and its efficiency is dependent on the rootstock genotype. Similar results have been reported in grafted cucumber (Liang et al. 2021). Nevertheless, Huang et al. (2016) reported that the bottle gourd rootstock grafting could not improve the watermelon performance under low Mg stress, while the improving effects can be found when grafted with pumpkin rootstock. This difference implies that suitable rootstocks should be carefully selected according to different nutrient deficiency environments. Moreover, we found that under 4 mM low-nitrate treatment, the growth of self-grafted watermelon plants slightly inhibited, but increased in rootstock-grafted plants relative to the 9 mM nitrate treatment. We suggest that bottle gourd rootstock grafting is expected to reduce N fertilizer application without compromising the plant growth of watermelon seedlings.

Bottle gourd rootstock-grafted watermelon plants have better root advantages in response to low-nitrate treatment

Root system plays a crucial role in adapting to changes in N availabilities (Rellán-Álvarez et al. 2016), and genetic components determine the fundamental morphology of plant roots (Pacheco-Villalobos and Hardtke 2012). Similarly, the results of our experiment showed that bottle gourd rootstock-grafted watermelon seedlings had larger root system than self-grafted seedlings, regardless of the N level (Fig. 1b, Table 3). The analysis of variance showed that graft combination significantly affected root morphological traits, and these parameters in ungrafted bottle gourd seedlings were significantly higher than those of ungrafted watermelon seedlings (Table 3, Figs. 2b and 4). These results indicated that rootstock-grafted watermelon plants had greater root advantage than self-grafted plants, which benefited from the genetic characteristics of bottle gourd rootstock. This may partly explain the superior growth of rootstock-grafted watermelon plants, since root morphological characteristics determine a plant's ability to acquire N which directly affecting the growth and development of plants (Jiang et al. 2017). The results of correlation analysis also showed that the growth parameters (including sound seedling index, root dry weight and whole dry weight) were positively correlated with root morphological traits (including total root length, root surface area, and root volume) (Fig. 11).

In addition, numerous studies showed that plant roots can signal the plant to alter the root system in response to various levels of N availability, so as to meet their own demand for N fertilizer (Pacheco-Villalobos and Hardtke 2012; Iqbal et al. 2020). In present study, the results showed that at 4 mM low-nitrate level, the total root length and root forks of bottle gourd rootstock-grafted seedlings and the total root length of ungrafted bottle gourd plants were increased, whereas these parameters of self-grafted seedlings decreased slightly (Table 3, Fig. 4a). The results showed that the bottle gourd rootstock could better adapt to low N through morphological variation.

NO₃⁻ uptake and transport of watermelon were improved by grafting onto bottle gourd rootstock under low-nitrate condition

Nitrate nitrogen (NO₃⁻-N) is one of the principal forms of available N in plants that determines the level of growth and development of plants. In this study, we found that regardless of the N level, the total NO₃⁻ content and NO₃⁻ uptake of rootstock-grafted watermelon seedlings were significantly higher than those of self-grafted seedlings (Fig. 5), and the NO₃⁻ uptake of ungrafted bottle gourd plants was also higher than that of ungrafted watermelon plants (Fig. 6), indicating that grafting with bottle gourd rootstock could improve the NO₃⁻ uptake of watermelon seedlings. Yang et al. (2013) also reported that bottle gourd rootstock-grafting can promote NO₃⁻ uptake in NaCl-stressed watermelon leaves. Genotypic variation of nitrogen utilization efficiency in oilseed rape showed that N-efficient genotype exhibited higher root N uptake than N-inefficient genotype (He et al. 2021). Furthermore, compared with 9 mM nitrate treatment, 4 mM low-nitrate treatment significantly reduced the total NO₃⁻ content and NO₃⁻ uptake of self-grafted plants, whereas increased the total NO₃⁻ uptake of rootstock-grafted plants, especially increased the NO₃⁻ content and NO₃⁻ uptake in the leaves of rootstock-grafted plants (Fig. 5). This result shows that bottle gourd rootstock grafting can improve the NO₃⁻ uptake and root-to-shoot transport of watermelon

seedlings under low-nitrate condition, which is in line with the results of Savvas et al. (2017), who found that nutrient uptake and translocation to the shoot were often improved in favourable grafting combinations. Correlation analysis showed that whole plant NO_3^- uptake was positively correlated with certain root morphological traits (Fig. 11), implying that the vigorous root systems of bottle gourd rootstock play an essential role in promoting the NO_3^- absorption and translocation of grafted watermelon seedlings. Martínez-Ballesta et al. (2010) reported that owing to the vigor of rootstocks, grafted plants usually show an increased uptake of water and minerals compared with self-rooted plants under favorable growth conditions. In addition, the total NO_3^- uptake of rootstock-grafted watermelon seedlings increased under 4 mM low-nitrate treatment but decreased in ungrafted bottle gourd seedlings, especially in the leaves (Figs. 5b and 6b). This may be attributed to the scion/rootstock interaction, which can be regulated by root-shoot-root long-distance signaling (Sasaki et al. 2014).

It is well known that the absorption of NO_3^- by plants is carried out through nitrate transporters (NRTs) located on the cell membrane of plant root epidermis and cortex. In superior plants, nitrate transporters include HATS and LATS that operate at low concentrations of NO_3^- -N (<1.0 mM) and above 1.0 mM, respectively, which are encoded by the NRT2 and NRT1 gene families, respectively (Bucher et al. 2014). A large number of studies have shown that NRTs play an important role in NO_3^- uptake and transport in many plants. However, these studies were mainly carried out on Arabidopsis (Zou et al. 2019; Jacquot et al. 2020) and field crops such as rice (Chen and Ma 2015), rape (Tong et al. 2020) and barley (Guo et al. 2020); the expression pattern and function of NRTs in vegetable plants are still less studied. Li et al. (2017) reported that the *AtNRT1.5* is expressed in the peri sheath of Arabidopsis root and is responsible for loading NO_3^- into xylem in root system. *AtNRT2.1* mediates root high-affinity NO_3^- influx of Arabidopsis roots and affects the transport of nitrate nitrogen to leaves (Li et al. 2007). In addition, NRT2 can regulate lateral root initiation in nitrate signaling pathway. In addition, NRT2.1 plays a direct stimulating role in the specific step of lateral root development, and can coordinate root development and external NO_3^- availability (Remans et al. 2006). In this study, the response of NRT gene expression in roots of self-grafted and rootstock-grafted watermelon plants to low nitrogen was quite different, the expression of *NRT1.5* and *NRT2.1* genes in roots of rootstock-grafted plants was significantly up-regulated under low-nitrate treatment, but there was no change in self-grafted plants (Fig. 7a and b). The bottle gourd rootstock-grafted plants had a higher ability to absorb NO_3^- than self-grafted plants. Therefore, the different expression levels of NO_3^- transporter gene may be part of the reason for the differences of NO_3^- absorption between self-grafted and rootstock-grafted watermelon plants, the NRT genes (*NRT1.5* and *NRT2.1*) may related in the higher NO_3^- uptake by rootstock-grafted plant roots. However, other NO_3^- transporter genes associated with high uptake of NO_3^- in rootstock-grafted plant roots need to be further studied.

N metabolism ability of watermelon was enhanced by grafting onto bottle gourd rootstock under low-nitrate condition

NO_3^- -N is absorbed into cells by plant roots and assimilated into amino acids and proteins through the reduction of a series of enzymes, such as NR, NiR, GS, and GOGAT (Xu et al. 2012). Among these enzymes, NR and GS are the rate-limiting enzymes of NO_3^- -N assimilation and play a crucial role in plants response to N deficiency (Kaur et al. 2015). NR and NiR convert nitrate to ammonium through the sequential reductive enzymatic action, and then the ammonium derived from nitrate reduction is further assimilated via the GS/GOGAT cycle. GS1 and GS2 are two isozymes of GS in cytoplasm and chloroplast, respectively. Zhang et al. (2017) reported that the NUE of cucumber could be enhanced by regulating the relative activity or expression of GS1/GS2. Yang et al. (2013) found that the increased N uptake and assimilation of rootstock-grafted watermelon in salinity stress is related to the enhancement of NR enzyme activity. Our previous research also showed that rootstock grafting can promote N metabolism of cucumber by increasing the activity of N metabolism enzymes (Liang et al. 2021). However, previous studies mainly focused on the physiological changes of enzyme activity, while little is known about the transcriptional expression of these enzyme related genes, especially in grafted plants. Therefore, the expression of N metabolizing enzyme genes in grafted watermelon leaves was studied. The results showed that *NR*, *NiR*, *GS1* and *GS2* genes in the leaves of bottle gourd rootstock-grafted plants were significantly up-regulated under 4 mM low-nitrate treatment relative to the 9 mM nitrate treatment, and were generally higher than those of self-grafted plants (Fig. 7c-f). The results proved that

under low-nitrate condition, bottle gourd rootstock grafting could enhance N metabolism ability of watermelon seedlings by regulating the transcriptional expression of N metabolizing enzyme genes. The correlation analysis showed that the relative expression of *NRT1.5* and *NRT2.1* genes, as well as the *NiR*, *GS1* and *GS2* genes were all positively correlated with each other (Fig. 11), implying that the NO_3^- metabolism in rootstock-grafted watermelon leaves was closely related to its absorption by roots and root-to-shoot transport.

Bottle gourd rootstock grafted plants have higher NUE and photosynthetic capacity under low-nitrate condition

After assimilation and metabolism, NO_3^- is gradually become incorporated into organic compounds, such as amino acids, proteins, and other N compounds necessary for plant growth and development (Pratelli and Pilot 2014). Consistent with the change of plant NO_3^- uptake, the N accumulation in roots, stems and leaves of bottle gourd rootstock-grafted plants was significantly higher than that of self-grafted plants, and increased under low-nitrate treatment, especially in the leaves (Fig. 8). The results indicated that watermelon grafted onto bottle gourd rootstock can not only absorb more N, but also transfer more N to leaves, so as to maintain high N accumulation and finally promote growth, which is agree with the view of Savvas et al. (2017). The correlation analysis also showed that there was a significant positive correlation between NO_3^- uptake, N accumulation and plant biomass (Fig. 11).

The ability of plants to absorb and utilization nitrogen can be evaluated according to two representative indexes: NUpE and NUtE. The NUpE and NUtE further jointly determine the overall NUE, and this role is influenced by the N supply (Garnett et al. 2015). Increasing the NUE is important to maintain a high productivity under low N supply (Xu et al. 2012). In general, plants have evolved a variety of mechanisms to increase N use (Bascuñán-Godoy et al. 2018). Efficient genotype have specific physiological mechanisms that enable them to absorb sufficient N and/or use the absorbed N more effectively. Similar results were discovered in our present study, which showed that the NUpE, NUtE, and NUE of bottle gourd rootstock-grafted plants were significantly higher than those of self-grafted plants (except of the non-significant in NUpE between self-grafted and rootstock-grafted plants under control treatment), regardless of the N level (Fig. 9), indicating that bottle gourd rootstock grafting is a very useful way to promote the NUE of watermelon seedlings though improving the NUpE and NUtE. Similar results were reported by previous studies (Yang et al. 2013; Nawaz et al. 2017; Sallaku et al. 2019). Furthermore, we found that 4 mM low-nitrate treatment remarkably increased the NUE traits of bottle gourd rootstock-grafted plants compared with control treatment, (Fig. 9). These results suggest the 4 mM nitrate concentrations in this experiment provided an appropriate level of N fertilization for bottle gourd rootstock-grafted watermelon seedlings, and appropriate N deficiency supply will not cause stress inhibition to the growth of watermelon seedlings, but may help to improve the NUE and promote the growth of watermelon seedlings (Chen et al. 2020).

In addition, photosynthesis is the main source of plant organic matter and participates in the synthesis of N-containing substances in chloroplasts. In turn, photosynthesis is also very sensitive to the change of N availability (Qin et al. 2018; Xu et al. 2015), because 57% of the N in the leaves is located in the chloroplasts, which is used to synthesize photosynthetic components and related enzymes (Xu et al. 2012). Therefore, there is a close coupling relationship exists between photosynthesis and N metabolism. In this study, we found that the Pn, Tr and chlorophyll contents of rootstock-grafted plants were all higher than those of self-grafted plants, and increased under 4 mM low-nitrate treatment, but decreased slightly in self-grafted plants (Fig. 10). The correlation analysis showed that the photosynthetic characteristics were positively correlated with N accumulation and NUtE (Fig. 11). The results indicated that watermelon grafted onto gourd rootstock can accumulate more N to enhance photosynthetic capacity. Furthermore, photosynthesis is related to the dry matter accumulation of plants, which is the basis for increasing crop growth, productivity, and NUE (Huang et al. 2016; Iqbal et al. 2020). The correlation analysis in this study proved that the photosynthetic characteristics were significant positive correlated with NUE and plant biomass (including plant height, shoot dry weight and whole dry weight) (Fig. 11).

Overall, correlation analysis showed that there were inextricably linked positive correlations among plant growth, root morphology traits, plant NO_3^- uptake, photosynthetic characteristics, NUtE and NUE (Fig. 11). Combined with the changes of

the above parameters in the text, we infer that grafting with bottle gourd rootstock may increase plant growth and NUE of watermelon seedlings by making better use of their developed roots and enhancing N metabolism potential and photosynthetic capacity to promote NO_3^- absorption, assimilation and metabolism. The significant positive correlation between the gene expression of nitrate transporters and N metabolizing enzymes further revealed that the enhanced NO_3^- uptake and N metabolism of rootstock-grafted plants were regulated at the transcriptional level. The results of this study provide key insights into how NUE could be improved under reduced N fertilizer application without compromising plant growth in the context of watermelon seedlings growth. As for mature plants, could be further studied in later experiments. Moreover, most previous studies have showed that there is chemical signaling from the root to the shoot, which plays an important role in regulating a plant's morphology and physiology (Tsutsui and otaguchi 2017). Therefore, it is necessary to further study the signal regulation pathway of grafting to promote watermelon plant growth and nitrogen use efficiency.

Conclusions

In conclusion, bottle gourd rootstock grafting can effectively promote the plant growth and NUE of watermelon seedlings, and the rootstock characteristics played a crucial role. The physiological mechanism of the improved performance of grafted watermelon under low N is related to the vigorous root systems and improved NO_3^- absorption capacity benefited from rootstock, and enhanced N metabolism potential in scion leaves. Significant up-regulation of nitrate transporter genes (*NRT1.5* and *NRT2.7*) and N metabolizing enzyme genes (*NR*, *NiR*, *GS1* and *GS2*) may be partially responsible for the improvements in NO_3^- uptake and metabolism of rootstock-grafted watermelon seedlings under low-nitrate treatment. Furthermore, rootstock grafting very likely to decrease the risk of yield loss of watermelon induced by inappropriate N supply, reduce inorganic N fertilizer waste, increase the efficiency with which resources are used, and reduce the cost of production without sacrificing plant growth. The 'Jingxinzen No.1' bottle gourd could be considered as a promising rootstock for plant grafting under low N conditions.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

This research did not involve human participants or animals.

Author Contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by Xiaoling Chen, Peijin Guo, Zhiyu Wang and Wenwen He. Jiayi Liang and Guohu Li analyzed the data. Xiaoling Chen wrote this manuscript. Ai zhen designed the experiment and revised this manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Plant growth (a) and root morphology (b) of grafted watermelon seedlings grown under 9 mM and 4 mM NO_3^- -N conditions. Z/Z: self-grafted watermelon seedlings; Z/J: rootstock-grafted watermelon seedlings

Figure 2

Plant growth (a) and root morphology (b) of ungrafted watermelon and bottle gourd seedlings grown under 9 mM and 4 mM NO_3^- -N conditions. Z: ungrafted watermelon seedlings; J: ungrafted bottle gourd seedlings

Figure 3

Root (a) and shoot (b) dry weight, sound seedling index (c), and R/S ratio (d) of ungrafted seedlings grown under 9 mM and 4 mM NO_3^- -N conditions. Values are mean $\pm$ SE ($n=3$). Different letters indicate significant differences determined by Tukey test at $P<0.05$. Results of a two-way ANOVA are indicated, * $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns, not significant. Z: ungrafted watermelon seedlings; J: ungrafted bottle gourd seedlings; N: N treatment; R: rootstock genotype; N×G: the interaction

Figure 4

Total root length (a), root surface area (b), root volume (c), Root forks (d) of ungrafted seedlings grown under 9 mM and 4 mM NO_3^- -N conditions. Values are mean $\pm$ SE ($n=3$). Different letters indicate significant differences determined by Tukey test at $P<0.05$. Results of a two-way ANOVA are indicated, * $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns, not significant. Z: ungrafted watermelon seedlings; J: ungrafted bottle gourd seedlings; N: N treatment; R: rootstock genotype; N×G: the interaction

Figure 5

NO_3^- content (a) and NO_3^- uptake (b) in the roots, stems and leaves of self-grafted and rootstock-grafted watermelon seedlings under 9 mM and 4 mM NO_3^- -N conditions. Values are mean $\pm$ SE ($n=3$). Different letters indicate significant differences determined by Tukey test at $P<0.05$. Results of a two-way ANOVA are indicated, * $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns, not

significant. Z/Z: self-grafted watermelon seedlings; Z/J: rootstock-grafted watermelon seedlings; N: N treatment; G: graft combination; N×G: the interaction; FW: Fresh weight

Figure 6

NO₃⁻ content (a) and NO₃⁻ uptake (b) in the roots and leaves of ungrafted seedlings under 9 mM and 4 mM NO₃⁻-N conditions. Values are mean ± SE (*n*=3). Different letters indicate significant differences determined by Tukey test at *P*<0.05. Results of a two-way ANOVA are indicated, **P*<0.05; ** *P*<0.01; *** *P*<0.001; ns, not significant. Z: ungrafted watermelon seedlings; J: ungrafted bottle gourd seedlings; N: N treatment; R: rootstock genotype; N×G: the interaction; FW: Fresh weight

Figure 7

Relative expression of nitrate transporter genes *NRT1.5* (a) and *NRT2.1* (b) in the roots, and nitrogen metabolism enzyme genes *NR* (c), *NiR* (d), *GS1* (e) and *GS2* (f) in the leaves of self-grafted and rootstock-grafted watermelon seedlings under 9 mM and 4 mM NO₃⁻-N conditions. Values are mean ± SE (*n*=3). Different letters indicate significant differences determined by Tukey test at *P*<0.05. Results of a two-way ANOVA are indicated, **P*<0.05; ** *P*<0.01; *** *P*<0.001; ns, not significant. Z/Z: self-grafted watermelon seedlings; Z/J: rootstock-grafted watermelon seedlings; N: N treatment; G: graft combination; N×G: the interaction

Figure 8

N accumulation in the root, stem and leaf of self-grafted and rootstock-grafted watermelon seedlings under 9 mM and 4 mM NO₃⁻-N conditions. Values are mean ± SE (*n*=3). Different letters indicate significant differences determined by Tukey test at *P*<0.05. Results of a two-way ANOVA are indicated, **P*<0.05; ** *P*<0.01; *** *P*<0.001; ns, not significant. Z/Z: self-grafted watermelon seedlings; Z/J: rootstock-grafted watermelon seedlings; N: N treatment; G: graft combination; N×G: the interaction

Figure 9

N uptake efficiency (a), N utilization efficiency (b) and N use efficiency (c) of self-grafted and rootstock-grafted watermelon seedlings under 9 mM and 4 mM NO_3^- -N conditions. Values are mean $\pm$ SE ($n=3$). Different letters indicate significant differences determined by Tukey test at $P<0.05$. Results of a two-way ANOVA are indicated, * $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns, not significant. Z/Z: self-grafted watermelon seedlings; Z/J: rootstock-grafted watermelon seedlings; N: N treatment; G: graft combination; N×G: the interaction; DW: Dry weight

Figure 10

Net photosynthetic rate (a), Transpiration rate (b) and Chlorophyll content (c) of self-grafted and rootstock-grafted watermelon seedlings under 9 mM and 4 mM NO_3^- -N conditions. Values are mean $\pm$ SE ($n=3$). Different letters indicate significant differences determined by Tukey test at $P<0.05$. Results of a two-way ANOVA are indicated, * $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns, not significant. Z/Z: self-grafted watermelon seedlings; Z/J: rootstock-grafted watermelon seedlings; N: N treatment; G: graft combination; N×G: the interaction

Figure 11

Pearson's correlation analysis between the NUE and the other parameters of the self-grafted and rootstock-grafted watermelon seedlings under 9 mM and 4 mM N conditions. *, ** and ***; Correlation is significant at the 0.05, 0.01 and 0.001 levels, respectively. NUE, nitrogen-use efficiency; SSI, sound seedling index; PH, plant height; rDW, root dry weight; sDW, shoot dry weight; wDW, whole plant dry weight; RL, total root length; RS, root surface area; RV, root volume; RF, root forks; NA, N accumulation; NUpE, nitrogen-uptake efficiency; NUE, nitrogen-utilization efficiency; NO₃⁻, whole plant NO₃⁻ uptake; Pn, net photosynthetic rate; Tr, transpiration rate; Chl, chlorophyll content; NRT1.5 and NRT2.1, nitrate transporters genes; NR, nitrate reductase gene; NiR, nitrite reductase gene; GS1 and GS2, glutamine synthetase genes