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Crossbreeding Rootstocks Improve Nitrogen Efficiency of Grafted Watermelon by Inducing Leaf Physiological and Root Morphological Responses

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Abstract: This is the first hydroponic study that evaluated the role of the heterotic plant characters of crossbreeding progenies and accessions which were used as rootstock for watermelon (scion) to improve the nitrogen (N) efficiency of this crop by grafting. The target of the research was to evaluate if grafting could enhance the nitrogen efficiency of watermelon through examining the responses of heterotic plant characters of crossbreeding rootstocks in the shoot development at the agronomical stage, root developments at the morphological stage, and leaf growth at various physiological stages. A hydroponic experiment was conducted by using an aerated deep-water culture (DWC) system in a well-equipped growth chamber of Erciyes University's Plant Physiology Laboratory located at Kayseri, Türkiye. A watermelon cultivar Crimson Tide (CT) was grafted onto watermelon cultivars of Calhoun Gray (CG), Charleston Gray (Cha. G), and accessions of PI 296341 and PI 271769, the crossbreed progenies of Calhoun Gray × PI 296341, Calhoun Gray × PI 271769, and Charleston Gray × PI 296341. Plants were grown in 8 L plastic containers filled continuously with aired stock nutrient solution under two nitrogen (N) doses (low dose N: 0.3 mM unit N, and high dose N: 3.0 mM unit N) in a completely randomized block design (RBD) which was replicated three times, for six weeks. The grafted plants usually showed a higher crop growth performance than the self-grafted control plants, illustrating that nitrogen efficiency was significantly enhanced with respect to rootstocks of crossbreed progenies under a low N dose and high N dose. The N efficiency of grafted watermelon (CT) was improved by the high manifestation of heterosis in some root morphological characters (vigor root development and active root mechanism) of some of the crossbreeding rootstocks (Calhoun Gray × PI 271769) particularly in low-N conditions. Additionally, some of the crossbreeding rootstocks (Charleston Gray × PI 296341) exhibited high heterosis, which led to improving the N efficiency of grafted watermelon (CT) by inducing leaf physiological responses under high N supply. This clearly indicated that heterosis plays a crucial role in exploiting the genetic diversity in the N efficiency of watermelon. Therefore, these heterotic plant traits may be vital for the selection and breeding of nitrogen-efficient rootstocks for both small-scale and large-scale commercial farming in the nearby future.

Keywords: backcrossing; *Citrullus lanatus* L.; N efficiency; root morphology; vegetable grafting

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1. Introduction

Sustainable food supply and agricultural production are critical for the survival of millions of households and communities worldwide since the world population is rapidly increasing at a rate of 77 million per annum. It is estimated to reach 8.5 billion by 2030, 9.7 billion by 2050, and 10.4 billion by 2100 [1]. Therefore, to fulfill the fast-growing food demand of the rising human population, food production must be increased by 70 percent from the current stage [2]. However, maximization of food production needs higher mineral fertilizer application. Nitrogen (N) is the most easily and broadly used fertilizer crop plants need for their development and growth. It is currently a key factor and vital input for crop developments, yielding small-scale and large-scale commercial farming.

Watermelon is among the most familiar cultivated fruits worldwide and of immense importance from an economic point of view. Regarding the reports by [3], China is the first (63.0 million tons), Iran is the second (4.1 million tons), and Türkiye is the third (4.0 million tons) largest producer country, which has a total contribution of 70.4% of the world's watermelon production (101 million tons). However, due to high fruit biomass production (42 tons/ha), a considerable dose of nitrogen fertilizers is needed for the yield and quality of this crop in Türkiye. However, unfortunately, to enhance yield, farmers usually apply a higher fertilizer dose than prescribed [4], and therefore the utilization of chemical N fertilizers across the world has escalated rapidly during the last forty years [2]. Nevertheless, the accessibility of nitrogen is often a restrictive factor for the growth of plants compared to other nutrients in terms of both high-input-rate and low-input-rate agriculture mechanisms [5].

Usually, in low-input agriculture systems, low amounts of N fertilizers are used mostly by peasant farmers, and thus the fertility of the soil is reducing, particularly among Asian and sub-Saharan African countries. Nevertheless, there are environmental issues related to increases in water and air pollution due to the overdosing of nitrogen fertilizers carried out by high-rate agriculture systems, particularly in EU countries and the USA [6]. However, the efficacy of N fertilizers is often low since, marginally, 30–40 percent of the fertilizer is assimilated and utilized by the crop plants [7], and the rest of the applied fertilizer is lost by way of leaching, volatilization, and also denitrification. As a result, minerals are lost from both the plant and soil mechanisms, leading to a rise in environmental pollution [2,8]. To secure the enhanced utilization of nitrogen availability in sustainable farming, enhanced fertilizers, as well as better crop and soil management practices, are needed [9]. With respect to these factors, the mechanism to enhance nitrogen efficiency and decrease production losses as a result of low nitrogen efficiency in high-yielding crop genotypes would be to graft them onto rootstocks with the ability to enhance the nitrogen efficiency of the scion in low-input-rate and high-input-rate crop systems [10].

Vegetable grafting is an innovative technology combining various scions and rootstocks in the horticultural area for the improvement of the biotic and abiotic stress tolerances of some Solanaceae and Cucurbitaceae species in Japan, China, Korea, and some parts of European and Asian countries [11]. Several studies were conducted to determine the significance of grafting to many abiotic stress tolerance systems of different crop plant varieties [10,12–21]. In vegetable production, grafting with efficient rootstock can enhance the N efficiency of plants [10,21–23] and depends on the genotypic variability of the scion section and the rootstock section with interactions of the scion/rootstock combination.

A broad spectrum of variability in fruit and vegetative traits is found in watermelon. To determine the inheritance of yield components in watermelon, breeding via heterosis is one of the most effective tools to find the genotypic variability in this crop [24]. However, it has been neither evaluated nor used in crop development programs resourcefully. Identifying and developing nutrient-specific rootstock and overcoming the deficiency of a particular nutrient is under the consideration of plant biologists [22,25]. Therefore, this research aimed to assess whether grafting could enhance the nitrogen efficiency of watermelon by examining the variation caused by the heterosis of crossbreeding rootstocks in the shoot growth at the agronomical stage, root growth at the morphological stage, and also leaf growth at the physiological stages.

2. Materials and Methods

2.1. Plant Material, Treatments and Experimental Layout

Hydroponic research was performed during the 2018–2019 farming season with the help of the aerated deep-water culture (DWC) technique in a regulated growth chamber of the Plant Physiology Laboratory for six weeks at Erciyes University's Faculty of Agriculture, Kayseri (location: Türkiye). Concerning vegetation growth, the average day/night temperatures were maintained at 25/22 °C, while the relative humidity was kept at 60–80%. The provided photon flux in the growth chamber was nearly 400 μmol m^{-2} S^{-1} with an

intensity of 16/8 h (light/dark) photoperiod accordingly. Watermelon cultivar of Crimson Tide (CT) was grafted onto watermelon cultivars of Calhoun Gray (CG), Charleston Gray (Cha. G), and accessions of PI 296341 and PI 271769, the crossbreeding progenies of Calhoun Gray × PI 296341, Calhoun Gray × PI 271769, and Charleston Gray × PI 296341. The seeds of the plant materials were sown in potted plastic trays, which contained a mixture of peat media (pH: 6.0–6.5) and perlite (2:1 ratio). Then, the appropriate seedlings were chosen for the grafting process by "cleft grafting," described by [26]. The scion was pruned to possess 1–3 true leaves, and the stem's lower part was cut to a slant angle to produce a tapered wedge. Later the scion was placed into the split made on the rootstock; a specifically devised grafting clip was used to bind the scion and rootstock together tightly. The self-grafted Crimson Tide was considered a control plant. Plants were allowed to heal and adapt for one week in a spacious container covered by double-layered plastic film and shade cloth in the climate chamber immediately after grafting [11]. To speed up recovery, the container was closed for the initial three or four days of the healing and adapting duration to inhibit grafted plants from wilting due to excessive transpiration. The plastic container was opened and closed for the following three or four days based on the grafted plants' conditions and the growing plants' space. This was needed to adapt grafted plants outside the plastic container to prevailing environmental conditions. After healing and acclimatization, grafted plants were relocated into 8 L plastic pots after roots were washed to remove growth media; every plastic pot was filled with nutrient solution and aerated by using an air pump (LP-100, 240 V/100 W, 0.042 mPa air pressures, 8400 L air hour^{-1}). As a result of relocating small seedlings into the solution culture, the solution culture was changed thoroughly in the initial two weeks and continuously every week.

The research was conducted in a completely randomized block design (CRBD) with three replicated blocks and three plants of every self-grafted cultivar and cultivar by rootstock combination in every block treated. During the renewal of the nutrient solution (one-week intervals), all nutrients were renewed when the nitrogen dose of the nutrient solution in the 3.0 mM N dose pots fell below 0.3 mM, as monitored daily with nitrate test strips (Merck, Darmstadt, Germany) with the help of a NitracheckTM reflectometer. This research used eight grafting combinations and two doses of nitrogen (Low dose N: 0.3 mM N, High dose N: 3.0 mM N). $Ca(NO_3)_2$ was utilized as a nitrogen source, and all other nutrients were kept similar. To avoid the Ca^{+2} imbalance in nutrient solution between low and high N treatments, $CaSO_4$ was applied (1.35 mM) only to the low N treatment. The stock solution used for the research is made of following components (µM): K_2SO_4 (500); $MgSO_4$ (325); KH_2PO_4 (250); H_3BO_3 (8); NaCl (50); Fe-EDDHA (80); $MnSO_4$ (0.4); $ZnSO_4$ (0.4); $MoNa_2O_4$ (0.4); $CuSO_4$ (0.4).

2.2. Harvest, Measurements of Shoot Dry and Root Dry Biomass

The growth of plants was measured with the help of three individual plants from every 8 L plastic container. The experiment was conducted under a completely randomized block design (CRBD) with three replicated blocks and three plants of every non-grafted cultivar and cultivar by rootstock combination in every block treated. Hence, the total measured plants were nine for each treatment. Shoot and root were separated into the leaf, stem, and roots to measure fresh weight. Harvested total plant fresh weight samples were measured with the help of a weighing scale, while the fresh root weight was weighed after drying the roots with tissue paper. Separated root parts were covered with a nylon pocket and kept in a cool room (5 °C) for root length measurement. While plant shoot samples were kept individually in paper bags and dried in an aerated oven at 70 °C for three consecutive days, the root samples were dried after root length determination.

The ratio of root/shoot was calculated by dividing the root dry weight over the sum of leaf and stem dry weights. The main stem length (cm) was measured with a ruler.

2.3. Calculation of N Efficiency Components

Three N efficiency components, N uptake, N use, and biological production efficiency (BPE) were determined from data acquired during harvest regarding formulas of [27,28]. At the time of analysis, each plant sample fraction was separately crushed with the help of an automatic mill. Right after crushing shoot dry materials, precisely 200 mg from each dry plant sample was collected to analyze the shoot nitrogen concentration (mg N g DW^{-1}) according to the Kjeldahl Nitrogen Determination procedure [29]. After analyzing the nitrogen concentration (mg N g DW^{-1}) in each plant fraction (leaf and stem) separately, the nitrogen content of each fraction was obtained by multiplying it by the dry weight of plant samples, respectively (g/plant). Nitrogen uptake efficiency was determined as the ratio of total nitrogen in the aboveground biomass to supplied nitrogen (mg N mg N^{-1}). NUE was determined as the total shoot dry matter to supplied nitrogen ratio (mg DW mg N^{-1}). BPE was determined as the total shoot dry matter to total nitrogen ratio in the aboveground biomass (mg DW mg N^{-1}).

2.4. Leaf Physiological and Biochemical Measurements

Every well-developed leaf was counted and recorded as a total leaf number (LN/plant). The plant samples' total leaf area (cm^2) was determined with the help of the leaf area measuring device LI-COR (LI-COR Model 3100, LI-COR. Inc., Lincoln, NE, USA).

Before harvesting, non-destructive measurements of the leaf-level CO_2 gas exchange (μmol CO_2 m^{-2} s^{-1}) were performed in a regulated growth chamber with the help of a portable photosynthesis system (LI-6400XT; LI-COR Inc., Lincoln, NE, USA). Photosynthetic measurement of a leaf (photosynthetically active radiation (PAR) = 1000 μmol m^{-2} s^{-1}, CO_2 at 400 μmol mol^{-1}) was conducted on the youngest widely developed leaves, using four replicate leaves in each treatment in the third and fifth weeks of the growth duration.

The Minolta SPAD-502 chlorophyll meter measured the leaf chlorophyll index (SPAD). At the time of the growth duration, fully developed leaves of whole plants for every treatment were measured once a week for data of SPAD. All SPAD readings were conducted between 09:00 and 12:00 a.m.

Nitrate reductase activity (NRA) in the leaf was measured based on the method suggested by [30]. Fresh plant samples were assembled and chopped into pieces; two grams of the latter were poured into every two falcon tubes and later labeled time-0 (T0) and time-60 (T60). The tubes were wrapped with aluminum foil to be protected from the light race. Ten ml of assay buffer solution (100 mM phosphate buffer, pH 7.5; 30 mM KNO_3; 5% (*v/v*) propanol) was poured onto every labeled tube (T0 and T60). The T0 container was quickly transferred into boiling water for five minutes, removed, and later allowed to cool at room temperature. Additionally, the T60 was maintained for 60 min at room temperature, transferred into boiling water for five minutes, and allowed to cool at room temperature. To detect nitrite in the assay tubes, the optical density (OD) of every standard tube was determined at 540 nm wavelength by using the spectrometer.

2.5. Measurements of Root Morphology

The plant root morphological parameters precisely; total root length (m), total root volume (cm^3), and average root diameter (mm) were determined with the help of the special image analysis software program WinRHIZO (Win/Mac RHIZO Pro V. 2002c Regent Instruments Inc., Québec, QC G1V 1V4, Canada) in conjunction with a recording device of Epson Expression 11000XL scanner (Long Beach, CA, USA).

2.6. Statistical Analysis

Statistical data analysis was conducted with the help of the PROC GLM procedure of the SAS Statistical Software (SAS for Windows 9.1, SAS Institute Inc., Cary, NC, USA). A two-factor analysis of variance was conducted to find the effects of various genotypes or grafting combinations and nitrogen and their interactions on the variables analyzed. The levels of significance were illustrated at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or n.s. as not

significant (F-test). Differences between the doses were analyzed using Duncan's multiple range tests ($p < 0.05$).

3. Results and Discussion

3.1. Biomass Accumulation and Partitioning

Results attained from the hydroponic experiment illustrated that shoot fresh weight and root fresh weight, stem length (Table 1), shoot dry weight, root dry weight, and –shoot ratio (Table 2) of grafted watermelon combinations were significantly ($p < 0.001$) affected by N rate, graft combinations and N rate x Graft combinations interaction. Irrespective of graft combinations, the plants grown under high nitrogen doses generally presented a higher performance in shoot and root growth and biomass production than plant crops cultivated under low nitrogen doses (Tables 1 and 2). Maximizing nitrogen supply from low to high doses led to a higher shoot fresh weight by approximately 248.3%, root fresh weight by approximately 58.3%, and main stem length by roughly 82.4% (Table 1). Additionally, similar increases were recorded in dried shoot matter accumulation by about 189.0% and root dry accumulation by almost 65.6%. However, the opposite result was found in the root–shoot ratio, which was practically a decline of 39.9% under a high N rate (Table 2). A higher root–shoot ratio under low N conditions is a characteristic plant response due to excessive dry matter partitioning into the root system. This can be stimulated by the roots' enhanced carbohydrate sink strength [10].

Table 1. Shoot fresh weight and root fresh weight and main stem length of self-grafted and grafted watermelon cultivars grown under low N dose (0.3 mM) and (3.0 mM) high N dose.

	Shoot Fresh Weight (g/plant)		**Root Fresh Weight (g/plant)**		**Main Stem Length (cm/plant)**	
Graft Combinations (S/R)	**Low N**	**High N**	**Low N**	**High N**	**Low N**	**High N**
CT						
CT/CT	13.8 i	37.8 g	6.2 j	7.6 i	30.3 g	48.5 ef
CT/PI 296341	19.9 h	62.7 e	10.6 g	19.5 a	38.8 efg	75.3 bc
CT/PI 271769	18.8 h	51.9 f	14.0 d	16.0 c	37.0 fg	75.5 bc
CT/CG	18.3 h	82.2 b	10.6 g	18.6 b	45.5 ef	85.3 ab
CT/(CG × PI 296341)	19.0 h	69.1 d	7.4 i	16.3 c	47.7 ef	88.2 ab
CT/(CG × PI 271769)	20.7 h	78.3 c	11.1 fg	18.5 b	54.0 de	98.3 a
CT/Cha. G	19.3 h	50.9 f	9.3 h	12.3 e	52.5 e	67.2 cd
CT/(Cha. G × PI 296341)	21.1 h	92.4 a	11.6 ef	19.0 ab	41.7 efg	95.3 a
F-Test						
Graft comb.	***		***		***	
N rate	***		***		***	
Graft comb. × N dose	***		***		**	

S: Scion, R: Rootstock, CT: Crimson Tide, CG: Calhoun Gray, Che. G: Chearleston Gray. [Z] Values represented by different letters shows significant differences between graft combination within both columns at $p < 0.05$. F values: ns, non-significant. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Our results confirmed that crop plants need copious quantities of nitrogen for better growth and high biomass production. This might result from a higher N uptake efficiency which might have contributed to a high leaf area development and thus a high photosynthetic and enzymatic (NRA) activity of leaves. The study by [10] examined the 10 local Turkish bottle gourd cultivars and two commercial watermelon varieties under high (3.0 mM) and low N rates (0.3 mM) during the screening experiment. In the second experiment, four gourd cultivars (N efficient: 70-07 and 07-45, N inefficient: 35-10 and 45-07) were chosen and used as rootstock for grafting with nitrogen inefficient watermelon cultivar (Crimson Sweet) under two different nitrogen rates. It was stated that significantly the highest shoot dry matter was found under high nitrogen dose compared to low nitrogen dose. Furthermore, maximizing nitrogen dose from low to elevated levels increased shoot dry matter by approximately 261%.

Table 2. Shoot dry weight and root dry weight and root: shoot ratio of self-grafted and grafted watermelon cultivars grown under low N dose (0.3 mM) and (3.0 mM) high N dose.

	Shoot Dry Weight (g/plant)		Root Dry Weight (g/plant)		Root: Shoot Ratio (g/g)	
Graft Combinations (S/R)	**Low N**	**High N**	**Low N**	**High N**	**Low N**	**High N**
CT/CT	1.05 h	2.78 f	0.12 h	0.23 g	0.11 d	0.08 e
CT/PI 296341	1.63 g	4.27 d	0.32 efg	0.70 a	0.19 b	0.16 c
CT/PI 271769	1.68 g	3.37 e	0.41 def	0.43 de	0.24 a	0.13 d
CT/CG	1.65 g	6.20 b	0.25 g	0.55 bc	0.15 c	0.09 e
CT/(CG × PI 296341)	1.55 g	4.42 d	0.25 g	0.47 cd	0.16 c	0.11 d
CT/(CG × PI 271769)	1.72 g	5.78 c	0.40 def	0.65 ab	0.23 a	0.11 d
CT/Cha. G	1.72 g	3.22 e	0.30 fg	0.38 def	0.17 c	0.12 d
CT/(Cha. G × PI 296341)	1.70 g	6.67 a	0.38 def	0.60 ab	0.23 a	0.09 e
F-Test						
Graft comb.	***		***		***	
N rate	***		***		***	
Graft comb. × N dose	***		**		***	

S: Scion, R: Rootstock, CT: Crimson Tide, CG: Calhoun Gray, Cha. G: Chearleston Gray. [Z] Values represented by different letters shows significant differences between graft combination within both columns at $p < 0.05$. F values: ns, non-significant. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Additionally, expected results have been illustrated by [31], who screened four hybrids and ten local Turkish genotypes, and four local Ghanaian tomato genotypes under high (3.0 mM) and low N rates (0.3 mM) in hydroponic experiments. The screening experiment confirmed that Helena F1 and ALT could be nitrogen efficient, while P005 and Karahidir are nitrogen inefficient tomato varieties. It was stated that plants grown under high nitrogen dose produced significantly highest shoot and root fresh and dry matter and main stem length than plants cultivated under low nitrogen dose under hydroponic systems.

Significant differences were reported among graft combinations in shoot and root growth, and the growth response to provided N, which is the interaction between N dose and graft combination, was also significant at a higher rate ($p < 0.001$) (Tables 1 and 2). The grafted plants enhanced their shoot and root growth and main stem length significantly more than self-grafted plants under low nitrogen and high N doses. In comparison to self-grafted plants, the grafting maximizes shoot fresh matter by approximately 41.5% and 84.08%, shoot dry matter by about 58.5% and 74.08%, root fresh matter by approximately 71.3% and 126.5%, the root dry matter by 182.7% and 131.6%, and main stem length by 49.4% and 72.3% at low nitrogen dose and high nitrogen dose, accordingly.

An efficient rootstock characterized by a strong and active root system can improve water and nutrient uptake from the soil solution and transfer to the aboveground of various plant parts [11]. All these results confirmed our hypothesis, suggesting that a low-yielding 'nitrogen-inefficient' watermelon genotype (Crimson Tide) can be enhanced when grafted onto 'nitrogen-efficient' crossbreeding rootstocks. This might result from high manifestation of heterosis in plant characters, mainly root morphological characters of some crossbreeding rootstocks (i.e., Calhoun Gray × PI 271769 and Charleston Gray × PI 296341). If so, all these indicated that heterosis plays a crucial role in exploiting the genetic diversity in the N efficiency of watermelon. In agreement with our study, comparable results were demonstrated in various grafting experiments such as with watermelon [10,32,33], melon [34], mini watermelon [35], tomato [31], and potato [20] under low and high N conditions. Considering the shoot fresh matter and dry shoot matter, watermelon plants grafted onto Cha's crossbreeding progeny at a high nitrogen rate (3.0 mM). G × PI 296341 performed relatively better than self-grafted plants, and the watermelon grafted onto other watermelon accessions and crossbreeding progenies.

Results in all perspectives also established our above hypothesis, which suggested that high manifestation of heterosis in crossbreeding rootstocks of Calhoun Gray × PI 271769 and Charleston Gray × PI 296341 led to improve N efficiency of watermelon (CT/CT).

However, at the low N rate (0.3 mM), no significant differences were found among the grafted watermelon plants compared with other accessions and crossbreeding progenies.

Concerning root fresh and dry matter, at the high N rate (3.0 mM), watermelon plants grafted onto the watermelon accession of PI 296341 produced significantly higher root fresh matter and root dry matter than self-grafted plants, and the watermelon grafted onto other watermelon accessions and crossbreeding progenies. However, the self-grafted watermelon plants observed considerably lower root fresh and dry matter. Nevertheless, at the low nitrogen dose (0.3 mM), significantly higher root fresh matter was produced when watermelon plants grafted onto the watermelon accession of PI 271769, though substantially higher root dry matter was produced when watermelon plants grafted onto the watermelon accession of PI 271769, the crossbreeding progenies of CG × PI 271769 and Cha. G × PI 296341.

Regarding root morphological results, a high manifestation of heterosis in crossbreeding rootstocks of Calhoun Gray × PI 271769 and Charleston Gray × PI 296341 existed and thus improved N uptake efficiency of watermelon (CT/CT) under low N conditions. Since, under the non-limiting N condition, nitrogen uptake is regulated by the demand of the growing crop [36]. In contrast, at limiting N, the N uptake depends on the extent and efficacy of the root system [37] and morphological root characteristics such as maximum rooting depth and root length density at deeper soil layers [27,38]. All these show that the appropriate rootstocks with a strong and active root system may easily contribute to the uptake of water and nutrients from the soil, thus increasing the uptake efficiencies of aboveground plant (scion) parts [11]. However, this might be enabled by maintaining assimilate allocation to the roots from vegetative tissues [37], which might be sustained by a greater leaf area and longer photosynthetically active leaf area duration. For instance, a grafting study that confirms our results indicated that the watermelon scion plants exhibited a 2.24-fold improvement in root dry weight when grafted onto pumpkin rootstock compared to the self-grafted control plants [39]. Corroborative results were demonstrated in several grafting studies conducted with tomato [31,40], mini watermelon [35], watermelon [10], eggplant [41], pepper [19], and potato [20] plants. They produced the significantly higher shoot and root biomasses than non-grafted plants under low and high N conditions.

3.2. N Uptake, N Use and Biological Production Efficiency

Results indicated that three N efficiency components—N uptake (NUp), N use (NUs), and biological production efficiency (BPE)—of watermelon plants grafted with different watermelon accessions and their crossbreeding progenies and self-grafted watermelon plants were affected ($p < 0.001$) significantly by nitrogen dose (Table 3). Irrespective of graft combinations, the plants grown under low nitrogen dose usually indicate a higher performance in nitrogen uptake, N use, and BPE than plants grown under high nitrogen dose. Maximizing N dose from the low to high stage led to a decline in N uptake by approximately 52.2%, in N use by about 71.2%, and in BPE by approximately 40.3%. These results confirmed that nitrogen fertilizers' efficiency is mostly low since watermelon plants usually assimilate less than 50% of the nitrogen provided [10].

Significant differences were reported among a combination of grafted plants in N uptake, N use, and BPE, and the effect of nitrogen provided, which is the interaction between N dose and graft combination, was also significantly higher ($p < 0.001$) (Table 3). Our results showed that the contribution of grafting to the N efficiency of watermelon (scion Crimson Tide) was significantly high. Compared with self-grafted plants, the grafting enhances nitrogen uptake efficiency by approximately 30.5% and 53.0%, N use efficiency by almost 58.5% and 73.6%, and BPE by nearly 22.1% and 12.7% under low N dose and high N dose, accordingly.

Table 3. Nitrogen uptake, nitrogen use, and biological production efficiencies of self-grafted and grafted watermelon cultivars grown under low N dose (0.3 mM) and (3.0 mM) high N dose.

	Nitrogen Uptake Efficiency (mg N/mg N)		Nitrogen Use Efficiency (g DW/g N)		Biological Production Efficiency (g DW/g N)	
Graft Combinations (S/R)	**Low N**	**High N**	**Low N**	**High N**	**Low N**	**High N**
CT/CT	0.542 e	0.224 i	31.25 b	8.28 f	57.62 f	36.91 k
CT/PI 296341	0.656 cd	0.307 h	48.61 a	12.70 de	74.07 b	41.31 hi
CT/PI 271769	0.755 ab	0.253 hi	50.10 a	10.02 ef	66.31 d	39.66 ij
CT/CG	0.637 d	0.419 fg	49.11 a	18.40 c	77.10 a	43.91 gh
CT/(CG × PI 296341)	0.690 bcd	0.311 h	46.13 a	17.21 de	66.85 d	45.08 hi
CT/(CG × PI 271769)	0.694 bcd	0.382 g	51.09 a	13.14 cd	73.72 b	42.31 g
CT/Cha. G	0.808 a	0.244 hi	51.09 a	9.33 ef	63.42 e	38.22 ijk
CT/(Cha. G × PI 296341)	0.714 bc	0.487 ef	50.60 a	19.84 c	70.82 c	40.73 hi
F-Test						
Graft comb.	***		***		***	
N rate	***		***		***	
Graft comb. × N dose	***		**		***	

S: Scion, R: Rootstock, CT: Crimson Tide, CG: Calhoun Gray, Cha. G: Chearleston Gray. [Z] Values represented by different letters shows significant differences between graft combination within both columns at $p < 0.05$. F values: ns, non-significant. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Among the graft combinations, the best performance in N uptake efficiency was shown by CT/Cha. G at low N conditions and by CT/(Cha. G × PI 296341) at high N conditions. This could result from a robust and active root-stock system that might contribute to water and nitrogen nutrient uptake. However, this confirms this hypothesis partially, but not completely. The grafts combinations of CT/Cha. G, CT/(CG × PI 271769), and CT/(Cha. G × PI 296341) showed almost the highest shoot dry matter production at low N (Table 2). Additionally, both graft combinations (CT/(Cha. G × PI 296341 and CT/(Cha. G × PI 296341)) showed the highest shoot dry matter, root dry matter, and root–shoot ratio under both low and high N supply, whereas the graft combination of CT/Cha. G exhibited the lowest results oppositely in shoot (at high N) and root parameters. Thus, the high N uptake efficiency of CT/Cha. G, only at low N, can be explained by a high leaf chlorophyll content (SPAD) of leaves that may contribute to photosynthesis. This indicates that the N uptake efficiency of both graft combinations (CT/(Cha. G × PI 296341 and CT/(Cha. G × PI 296341)) is closely associated with the root morphological characteristics. Regarding root morphological results (Table 2), a high manifestation of heterosis in cross-breeding rootstocks of Calhoun Gray × PI 271769 and Charleston Gray × PI 296341 existed and thus improved N uptake efficiency of watermelon (CT/CT) under low N conditions. This also confirmed that the interaction between supplied nitrogen and graft combination was highly significant, which might be due to responses of heterotic plant root characters.

Similar interactions were found in N use efficiency since the graft combination of CT/(CG × PI 271769) exhibited the highest NUE at low N, while at high N conditions, the highest NUE was shown by CT/(Cha. G × PI 296341). The highest BPE was shown by CT/CG and CT/(CG × PI 271769) graft combinations at low and high N conditions, respectively. Again, all these results confirmed that our hypothesis, which proposes that a low yielding 'N-inefficient' watermelon cultivar (Crimson Tide) can be improved when it is grafted onto 'N-efficient' crossbreeding rootstocks (i.e., (CG × PI 271769) and (Cha. G × PI 296341)). Regarding these results, a high manifestation of heterosis in cross-breeding rootstocks of Calhoun Gray × PI 271769 and Charleston Gray × PI 296341 existed and thus led to improved NUE of watermelon (CT) under both low and high N conditions.

In corroboration with our study, comparable results were demonstrated in various grafting experiments such as with watermelon [10,32,33], melon [34], mini watermelon [35], tomato [31], and potato [20] under low and high N conditions.

3.3. Total Leaf Area, Total Leaf Number, Intensity of Photosynthesis Measurements, Leaf Chlorophyll Content (SPAD) and Leaf NRA Activity

The results indicate that total leaf area, total leaf number, duration of photosynthesis measurements, leaf chlorophyll content (SPAD), and leaf NRA activity were affected significantly ($p < 0.001$) by N rate, graft combinations, and the interaction of graft combination × N rate (Figure 1 A–E). Regardless of grafting, watermelon plants under high nitrogen dose exhibited mainly an enhanced performance regarding total leaf area, total leaf number, the intensity of photosynthesis measurements, SPAD, and leaf NRA activity compared to plants grown hydroponically under low nitrogen dose.

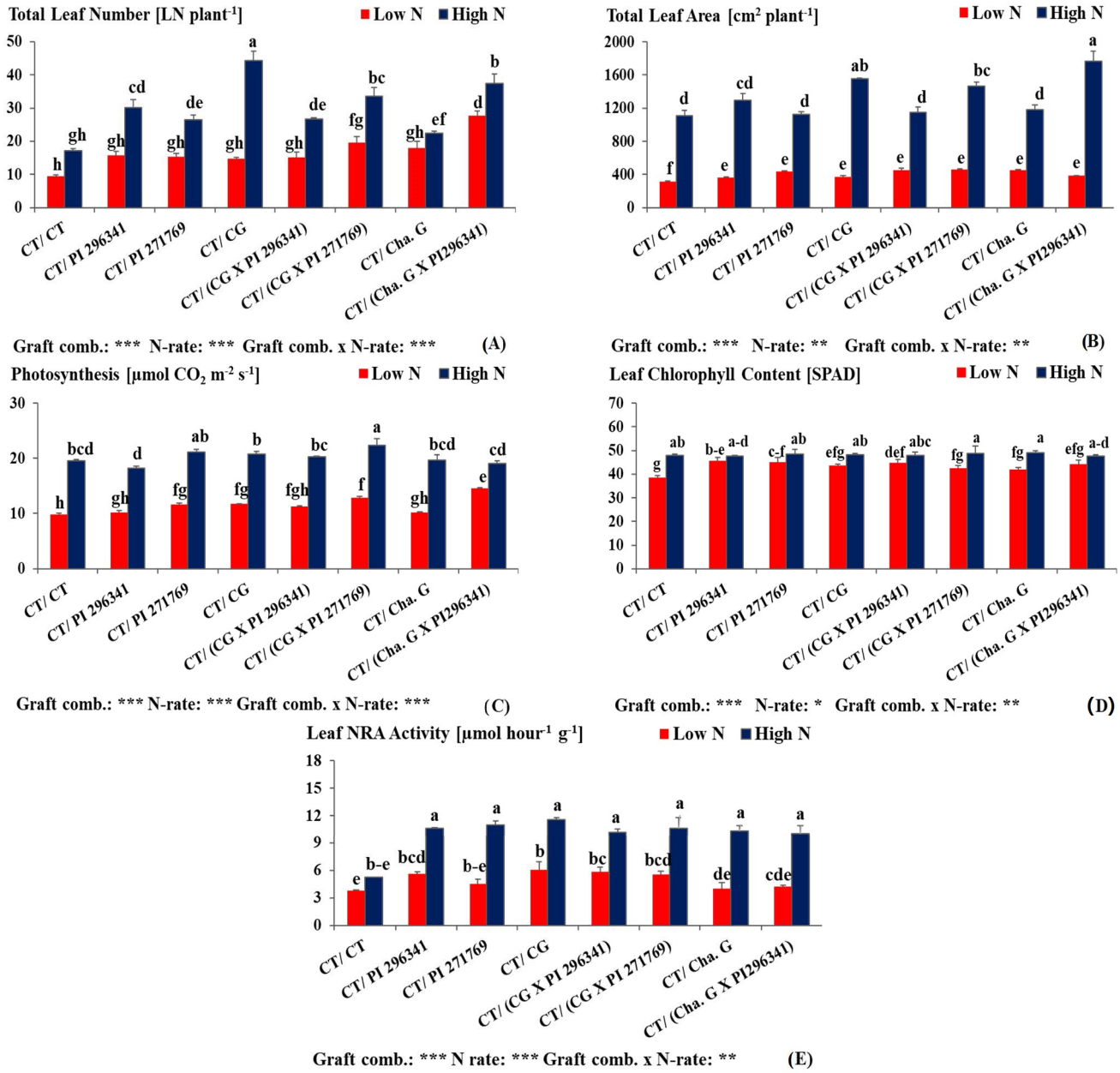


Figure 1. Total leaf number (**A**), total leaf area (**B**), the intensity of photosynthesis measurements (**C**), leaf chlorophyll content (**D**), and leaf NRA activity (**E**) of self-grafted and grafted watermelon cultivars grown under low N dose (0.3 mM) and high (3.0 mM) N dose. CT: Crimson Tide, CG: Calhoun Gray, Che. G: Chearleston Gray, [Z] Values represented by different letters shows significant differences between graft combination within both columns at $p < 0.05$. F values: ns, non-significant. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Increasing nitrogen dose enhanced the total leaf number by approximately 75.9%, the total leaf area by 230.4%, the intensity of photosynthesis measurements by 74.3%, SPAD by 11.2%, and leaf NRA activity by 101.4% at high N conditions. The study by [19] stated that increasing nitrogen dose from low to a high level in the stock nutrient solution enhanced pepper inbred lines in dried shoot matter by approximately 151%, shoot nitrogen assimilation by approximately 366%, shoot N concentration by approximately 86%, total leaf area by approximately 227%, photosynthesis by approximately 20%, and leaf NR activity by approximately 282%. All of these indicated that nitrogen has a significant positive impact on leaf area formation. Thus, the photosynthetic activity of leaves when it is supplied to the plants is non-limited; its uptake is usually regulated by the growing demand for the crop [36].

In the study of [20], two potato cultivars (Agria: N efficient, and Van Gogh: N inefficient) were grafted in a reciprocated manner onto each other, after which it was examined under two N doses (Low N dose: 0.5 mM N, and High N dose: 3.0 mM N) with the help of deep-water culture (DWC) system. Maximizing the N dose improved the total leaf number by approximately 24.3%, the total leaf area by 98.8%, and the intensity of photosynthesis measurements by 34.4% under a high nitrogen dose. According to some previous studies, under a high N rate, total leaf area (183%) also and the intensity of photosynthesis measurements (67%) increased substantially under hydroponic conditions in watermelon [10]. Our results indicated that nitrogen impact on crop plants has positive correspondence on plant shoot growth that may have supported a more significant leaf area development and thus a more significant enzyme (NRA) and photosynthetic activity of leaves [10,31,41].

Considering the total leaf number, at the high N rate (3.0 mM), watermelon plants grafted onto the accessions of CG performed better than self-grafted plants, and the watermelon grafted precisely onto other watermelon accessions and crossbreeding progenies. Again, all these results confirmed our hypothesis, suggesting that a low yielding 'nitrogen inefficient' watermelon genotype (Crimson Tide) can be enhanced when grafted onto 'nitrogen-efficient' crossbreeding rootstocks. This might result from the high manifestation of heterosis in plant root morphological characters of some crossbreeding rootstocks (i.e., Calhoun Gray × PI 271769 and Charleston Gray × PI 296341), particularly at low N conditions. Although CT/CG showed the highest leaf number at high N, CT/(Cha. G × PI 296341) exhibited the highest leaf number under low N conditions; this could result from a high N allocation from root to shoot; an efficient rootstock which is characterized by a strong and active root system can improve water and nutrient uptake from the soil solution, and transfer to aboveground of various plant parts [11].

Related results have also been reported by [41]. The study was conducted hydroponically by using 14 different eggplant genotypes from Türkiye's genetic resources (*Solanum melongena* L.) under high (3.0 mM) and low N (0.3 mM) rates for screening. Results of the screening experiment confirmed that Kemer and Yıldırım could be characterized as N efficient, while Adana Dolmalık and Manisa as N-inefficient eggplant varieties. It was stated that plants cultivated under high N dose significantly produced the highest total leaf number than those cultivated under low N dose under the hydroponic conditions. Familiar findings have also been stated in different grafting research in tomatoes [31] and potatoes [20]. On the other hand, at the low N rate (0.3 mM), a significantly higher total leaf number was produced when watermelon plants were grafted onto the crossbreeding progenies of Cha. G × PI 296341.

Regarding total leaf area, at the high N rate (3.0 mM), watermelon plants grafted onto the crossbreeding progenies of Cha. G × PI 296341 produced significantly higher total leaf area than control plants, and the watermelon grafted onto other accessions and crossbreeding progenies. However, at the low N rate (0.3 mM), no significant differences were observed among grafted watermelon plants compared with other accessions and crossbreeding progenies. Our results were in line with the study of [10], who stated that grafting enhanced the total leaf area by approximately 78% and 336%, respectively, at low

and high N rates at the watermelon plants. Comparable results were studied in grafted mini watermelon [34], tomato [31], eggplant [41] and potato [20].

At high N conditions, the duration of photosynthesis measurements of watermelon grafted onto the crossbreeding progenies of CG × PI 271769 was extensively more elevated than the self-grafted watermelon plants. Nevertheless, at a low nitrogen rate, the duration of photosynthesis measurements of watermelon grafted precisely onto most of the rootstocks was enhanced; furthermore, a higher significant value was found at the crossbreeding progenies of Cha. G × PI 296341. This might be due to efficient N allocation contributed by roots (rootstock) to shoot (scion) that lead to an increase in leaf area and the number of leaves, hence raising the photosynthesis. In the study of [33], wild watermelon rootstocks enhanced the development and photosynthetic rate of the watermelon plants compared to self-grafted ones. The leaf structure, the number of cells, mesophyll thickness in the palisade parenchyma, spongy parenchyma, and intercellular distances were altered by the rootstocks [32]; so, as a result, the photosynthetic assimilation was enhanced. Grafting potatoes reciprocally improved the total leaf area, total leaf number, and the duration of photosynthesis measurements [20].

In plant crops, chlorophyll is a green pigment vital for photosynthesis; it helps transform light energy into chemical energy during photosynthesis. Additionally, the amount of chlorophyll in a leaf is crucial to depict plants' growth [42]. Therefore, in crop production, chlorophyll is necessary for photosynthetic activities. Concerning SPAD, at the high N rate (3.0 mM), a significantly higher value was observed in watermelon plants grafted onto the crossbreeding progenies of CG × PI 271769 and watermelon accession of Cha. G than control plants. We also observed that increase in total leaf area, total leaf number, leaf SPAD, and photosynthetic activities corresponded with an increase in shoot biomass (Tables 1 and 2). Similarly, the chlorophyll contents of the N-inefficient tomato genotype of Karahidir were significantly improved by grafting precisely onto N-efficient tomato rootstocks of Helena F1 and Alt than non-grafted plants under a high N rate (3.0 mM) [31]. The structure of the leaf involves the photosynthetic rate of leaves. The leaf mesophyll conductance is impacted by the thickness and structure of the spongy parenchyma and palisade parenchyma [32]. Pumpkin rootstock enhanced the average chlorophyll content and intensity of photosynthesis of watermelon by causing the expression of the chlorophyll generated genes and declining the expression of the chlorophyll deteriorated genes [32,43].

Rootstocks enhance nutrient and cytokine supply to the scion, enhance growth and dry matter accumulation, and initiate the gene expression of nitrate reductase enzymes [22,32,43,44]. Results of leaf NR activity indicate that grafted watermelon plants recorded highest leaf NR activity than self-grafted plants, though there were no significant differences observed among them under a high N rate. Thus, leaf NR activity increases, leading to grafted plants' increased N uptake capability [41]. Our findings were similar to those in the study by [35], which observed that NR activities in grafted mini-watermelon plants were higher than in non-grafted plants, positively affecting efficient N uptake and N utilization for shoot biomass production.

All these results indicated that grafting with the crossbreeding progenies, particularly with Calhoun Gray × PI 271769 and Charleston Gray × PI 296341, led to an increase in some leaf physiological (total leaf area, total leaf number, photosynthesis) responses of Crimson Tide (scion) which might be associated with an efficient N allocation contributed by roots (rootstock) to shoot (scion) that lead to increase in leaf area and the number of leaves and hence raise the photosynthesis. An efficient rootstock characterized by a strong and active root system can improve water and nutrient uptake from the soil solution and transfer to the aboveground of various plant parts [11]. Therefore, nitrogen uptake and nitrogen use efficiencies of watermelon (CT) were significantly improved under high N conditions (Table 2).

3.4. Total Root Length, Total Root Volume and Average Root Diameter

Total root length and root volume were affected significantly ($p < 0.001$) by nitrogen dose, though there were no significant differences found concerning average root diameter (Table 4). Irrespective of graft combinations, plants grown hydroponically under high nitrogen dose generally showed enhanced performance in developing root morphology than plants grown hydroponically under low nitrogen dose. Enhancing N dose enhanced total root length by approximately 110.0%, though a decline in root volume by 10%.

Table 4. Total root length, root volume and average root diameter of self-grafted and grafted watermelon cultivars grown under low N dose (0.3 mM) and (3.0 mM) high N dose.

	Total Root Length (m/plant)		**Total Root Volume (cm^3/plant)**		**Av. Root Diameter (mm)**	
Graft Combinations (S/R)	**Low N**	**High N**	**Low N**	**High N**	**Low N**	**High N**
CT/CT	313.2 i	1010.3 g	191.0 ef	187.3 ef	0.284 fgh	0.345 b
CT/PI 296341	702.1 h	3500.1 a	112.0 g	245.3 d	0.297 e	0.258 hi
CT/PI 271769	861.1 gh	1871.1 de	118.0 f	244.3 d	0.313 d	0.324 c
CT/CG	710.0 h	1665.6 ef	770.0 a	312.0 c	0.284 fg	0.414 a
CT/(CG × PI 296341)	845.9 gh	2069.3 d	213.7 e	266.3 d	0.324 cd	0.274 hi
CT/(CG × PI 271769)	1869.2 de	2829.4 b	392.7 b	248.0 d	0.350 b	0.320 cd
CT/Cha. G	938.0 g	1499.6 f	210.0 ef	225.0 e	0.287 ef	0.276 ghi
CT/(Cha. G × PI 296341)	1737.4 e	2302.8 c	329.0 c	378.7 b	0.353 b	0.267 ij
F-Test						
Graft comb.	***		***		***	
N rate	***		***		n.s.	
Graft comb. × N dose	***		***		***	

S: Scion, R: Rootstock, CT: Crimson Tide, CG: Calhoun Gray, Che. G: Chearleston Gray. [Z] Values represented by different letters shows significant differences between graft combination within both columns at $p < 0.05$. F values: ns, non-significant. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Our results were similar to that of [32] that grafting showed enhanced shoot and root biomass production in watermelon and pumpkin grafting combination and enhanced nitrogen efficiency in grafted plants. Familiar findings were reported in grafted tomato [31], grafted watermelon [10], grafted eggplant [41], grafted pepper inbred lines [19], and grafted potato [20].

Total root length, root volume, and average root diameter were affected significantly ($p < 0.001$) by graft combinations and the interaction of graft combination × N dose (Table 3). Compared with self-grafted plants, the grafting enhanced the total root length by approximately 249.5% and 122.5%, and the total root volume by approximately 60.5% and 46.3% at low N dose and high N dose, accordingly.

Compared to self-grafted plants, the highest total root length was produced when watermelon was grafted onto accession of PI 296341 at high N rates. Nevertheless, under low N doses, significantly the highest total root length was generated when watermelon was grafted onto the crossbreeding progeny of CG × PI 271769. Moreover, significantly the most total root volume was generated under high nitrogen rates when watermelon was grafted onto the crossbreeding progeny of Cha. G × PI 296341, though under low N rate accession of CG, significantly produced the highest root volume when used as a rootstock.

Concerning average root diameter, a significantly greater value was observed under a high nitrogen rate when the accession of CG was used as a rootstock. Nevertheless, a substantially greater value was observed under a low nitrogen dose when watermelon was grafted onto the crossbreeding progeny of Cha. G × PI 296341. Our results were in line with several grafting studies such as tomato [31], potato [20], and eggplant [41] under high nitrogen dose in comparison to low nitrogen dose. However, opposite to our result, the study by [10] reported that total root length and total root volume of graft combinations showed significant differences ($p < 0.001$), but were negatively affected by the different doses of nitrogen provided. In contrast to nitrogen impact, grafting recorded a significantly

positive impact on root morphology and henceforth enhanced (maximum) the total root length by approximately 65% and 58% and root volume by approximately 183% and 211% at low and high nitrogen doses, accordingly, as compared to self-grafted watermelon plant crops. These outcomes can be explained by the fact that the impact of grafting on the scion is affiliated with the root potential of the rootstock.

Regarding root morphological results, the crossbreeding progeny Calhoun Gray × PI 271769 consistently exhibited significantly vigorous root and vibrant root mechanism growth, specifically under low nitrogen doses. This might result from the high manifestation of heterosis in root morphological characters of this crossbreeding progeny since most increased biomass production (Tables 1 and 2), and highest NUE (Table 3) was also shown by Calhoun Gray × PI 271769, particularly under low N conditions. Consequently, the N efficiency of grafted watermelon (CT) was improved by high manifestation of heterosis in some root morphological characters (vigor growth and active root system) of the crossbreed rootstocks Calhoun Gray × PI 271769, specifically at low nitrogen dose.

4. Conclusions

This study investigated whether grafting could improve the nitrogen efficiency of watermelon by examining the variation caused by heterosis of crossbreeding rootstocks under two contrasting N conditions. The obtained results via investigations on the shoot growth at the agronomical stage, root growth at the morphological stage, and leaf growth at the physiological stages indicated that crossbreeding rootstocks improved the nitrogen efficiency of grafted watermelon. The watermelon plants grafted with different crossbreeding rootstocks usually showed a higher crop growth performance than self-grafted control plants, although significant variations were found between the graft combinations. This resulted from the high manifestation of heterosis in plant characters, particularly root morphological characters of some crossbreeding rootstocks (i.e., Calhoun Gray × PI 271769 and Charleston Gray × PI 296341). Based on the high manifestation of heterosis, the crossbreeding progenies of Charleston Gray × PI 296341 can be used as N-efficient rootstocks under high N conditions. On the other hand, the N efficiency of grafted watermelon (CT) was improved by high manifestation of heterosis in some root morphological characters (vigor growth and active root system) of the crossbreed rootstocks Calhoun Gray × PI 271769, particularly at low N conditions. All these vividly indicated that heterosis plays a crucial role in exploiting the genetic diversity in the nitrogen efficiency of watermelon. Therefore, these heterotic plant traits could be necessary for selecting and breeding nitrogen-efficient rootstocks for both low-input and high-input farming in the near future. Nevertheless, the responsiveness of these rootstocks on reproductive character and fruit quality factors needs to be examined for further investigations.

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