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Natural variation in response to nitrate starvation among varieties of habanero pepper (*Capsicum chinense* Jacq.)

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Abstract

Habanero pepper (*Capsicum chinense* Jacq.) is one of the most cultivated plants in the world. We evaluated the physiological and molecular behavior of three habanero pepper varieties seedlings, two from the Yucatan region (Mayan Ek and Mayan Chan) and one commercial variety (SEMINIS), subjected to 10 days of nitrogen (N) deficiency treatment. Growth parameters, N compounds, net nitrate absorption, and *CcNRT2.1* (*Capsicum chinense* high affinity nitrate transporter) gene expression were evaluated at 0, 2, 6, 8 and 10 days of the treatment. Mayan Ek presented a higher total dry mass (0.266 ± 0.002 g) during treatment period, when compared to the other varieties (0.167 ± 0.002 g and 0.106 ± 0.001 g for Mayan Chan and SEMINIS, respectively). The N metabolite contents suggest that the adaptive mechanism in Mayan Ek involves a re-mobilization of N reserved when a deficit exists, mainly from root nitrate and amino acids from other parts of plant. Moreover, total amino acid content may be determinant in the final dry biomass for this species, regardless of variety and the degree of exposure to N ($R^2 = 0.89$). Mayan Chan showed a higher high-affinity nitrate uptake when exposed to N deficit (8.22 ± 0.69 µmoles g^{-1} DW min^{-1}); thereby, revealing a different adaptive mechanism. All varieties increased their transcript levels of *CcNRT2.1* gene, but differed in their induction period. We identified significant morphological and molecular traits that may increase the productivity of habanero pepper growing on soils with restricted N phyto-availability.

Keywords: Adaptative response; *Capsicum chinense* Jacq.; high affinity nitrate uptake; nitrogen metabolites; nitrogen starvation; plant growth; remobilization.

Abbreviations: *CcNRT2.1*_high affinity nitrate transporter of *Capsicum chinense*; N_nitrogen; NUE_nitrogen use efficient; $\bar{R}$ - mean value of relative growth rate.

Introduction

Nitrogen (N) is a constituent of many important cellular compounds, such as amino acids, proteins (enzymes and structural proteins), nucleic acids (DNA and RNA), adenosine triphosphate (ATP), chlorophyll and some phytohormones (auxin and cytokinins). In addition, the concentration of this element is considered to be an indirect determinant of the photosynthetic rate, biomass accumulation and yield, because it is essential for the chlorophyll biosynthesis and the enzymes that fix CO_2 during photosynthesis (Evans, 1983). It has been reported that the worldwide doubling of agricultural production over the past four decades is associated with a sevenfold increase in the use of N fertilizers (Hirel et al., 2007). This value is expected to increase by approximately three times by 2050 (Good et al., 2004). The need to apply N for optimal crop production is related to the fact that the phytoavailability of this element cannot supply the quantities that are required for the rapid growth of crops in the vast majority of agricultural land (White and Brown, 2010). However, there are two current major challenges to the application of greater nitrogen fertilizer to crops. The first challenge is the negative environmental impact caused by the excessive application of nitrogenous fertilizers. The N leaching and phosphorus losses are the main culprits in eutrophication processes in water (Conley et al., 2009). In addition, N fertilizers are the largest source of greenhouse gas emissions in agriculture (Galloway et al., 2008). The other challenge to the continued use of inorganic N fertilizers would be the large increase in fertilizer prices, which is not agriculturally sustainable (White and Brown, 2010; Anbessa and Juskiw, 2012). An alternative method to achieve sustainable agriculture is to develop and/or grow varieties that use N (NUE) efficiently. The NUE is defined as the yield (grain, fruit or foliage) per unit N available in the soil (Kant et al., 2011). To achieve this goal, it is necessary to have a throughout understanding of the regulatory mechanisms that control the NUE, particularly when this mineral element is limited. The NUE is a complex parameter and regulated by different genes (Gallais and Hirel, 2004) and involves different stages, which include the uptake, assimilation, translocation, recycling and remobilization of N. The improvement of each of these features is very specific for each type of plant (Chardon et al., 2012), which is why the improvement of each individual crop requires a comprehensive understanding of the different stages mentioned above and the knowledge of each plant species with regard to its N metabolism.

The genetic variability that allows different plant species to grow under N deficient conditions is of scientific value and can provide an understanding of the processes that regulate NUE and understanding how to develop cultivars with better N usage in soils with low contents of this element (Richard-Molard et al., 2008; Ikram et al., 2012; Shi et al., 2010). In a study of two rice varieties with differing field NUE, Shi et al. (2010) suggested that the high affinity nitrate transporter OsNRT2.1 has an important role in the acquisition of N in this species by partially regulating N uptake, growth and root development. It has been reported that N utilization efficiency appears to be of major importance when barley plants are grown under low N, when compared with the uptake efficiency of this element (Beatty et al., 2010). Beatty et al. (2010) observed differences in the amino acid contents between barley genotypes that could be related to the efficient use of N in these genotypes.

Recently, variations in growth responses to the limitation and nitrate deficiency of 23 *Arabidopsis* accessions were studied by evaluating different morphological and metabolic traits (Ikram et al., 2012). These authors characterized different adaptive responses between *Arabidopsis* ecotypes, which allowed the plants to tolerate an imbalance in the exogenous N supply. These adaptive responses were grouped into four classes, depending on the N conditions to which were exposed. It was shown that the growth of this species was regulated by key traits, such as the shoot to root nitrate content ratio, root fresh weight and root amino acid content, and shoot fresh weight together with root thickness (Ikram et al., 2012).

Most of the studies described above aimed at understanding plant responses to an N deficiency and were carried out by comparing the responses of plants subjected to N deficiency versus plants under non- limiting N (Ding et al., 2011; Krapp et al., 2011). This type of study allows us to understand in detail how these processes occur, but not the importance they may have in the adaptation of plants to N availability. The genus *Capsicum*, which is native to subtropical and tropical America, is one of the most cultivated plants in the world and plays a key role as a constituent of many food products (Wang and Bosland, 2006). In addition, *Capsicum* has a high nutritional value, is an excellent source of vitamins C, A and B complex, and minerals such as folate, potassium and molybdenum manganese (Kothari et al., 2010). It is known that habanero pepper, such as any other crop, is vulnerable to N shortages, which reduce flower and fruit production as well as the contents of capsaicinoids in the fruit (Medina-Lara et al., 2008; Monforte-González et al., 2010). However, there is no detailed study of the response of this species to N deficient conditions. In the present study, we investigated the growth response to a deficit of nitrate, which is the main source of N in agricultural soils, in three different varieties of habanero pepper (*Capsicum chinense* Jacq.). Our aim was to identify contrasting responses that might represent different adaptive strategies by this species to these conditions.

Results

Growth differences between habanero pepper varieties subjected to N deficiency

We analyzed changes in fresh weight and dry biomass production for three habanero pepper varieties grown under hydroponic and N deficient conditions. Fig 1 shows that there were growth differences between the three varieties when they were subjected to this treatment. Variety Mayan Ek showed the highest fresh and dry weight values by the tenth day of treatment, both for the aerial parts (Figs. 1A and 1B) and the roots (Figs. 1C and 1D).

Although the fresh weight of the aerial parts was different at the beginning among the varieties, variety Mayan Ek nearly doubled its initial weight by the end of the experiment, and the Mayan Chan and SEMINIS varieties increased by 1.4 to 1.7 times, compared with their initial weight, respectively (Fig. 1A). However, the difference in the dry weights of the aerial parts was even greater towards the end of the experiment, when they were approximately 4, 2 and 3 times that of its initial weight for Mayan Ek, Mayan Chan and SEMINIS, respectively (Fig. 1B).

Mayan Ek also produced greater root growth under deficit conditions when compared with the other two varieties, which showed a gain of approximately 5 and 4 times the fresh weight and dry matter production, respectively. The gains in the other varieties were similar with approximately 3 and 1.7 times for fresh and dry weight, respectively (Figs. 1C and 1D).

On the other hand, the Mayan Chan variety had higher fresh and dry weights for the shoots and roots on the tenth day of treatment, when compared with the SEMINIS (Fig. 1). However, the gain in both weights during the deficit period was similar in both varieties and in some cases was slightly higher in SEMINIS. The data at the beginning of the experiment, which were always superior for Mayan Chan compared with SEMINIS, appeared to explain this behavior (Fig. 1).

All varieties reached a higher total dry weight when grown in the presence of 1 mM KNO_3 (control) for 10 days in comparison to the N deficit treatment (Fig. 2A). These data demonstrate that although the dry weight of both plant parts was increased during the deficit period, they are still affected by this condition. Again, higher values were obtained for the Mayan Ek variety, with 1.6 and 2.2 times higher numbers than those found for the Mayan Chan and SEMINIS varieties, respectively.

Additionally, significant differences in the root/shoot dry weight ratio were observed between the different exposures to N deficiency and between varieties, reflecting differences in growth rates between the two parts of the plant (Fig. 2B). The Mayan Ek variety exhibited a significantly reduced ratio beginning on the second day of treatment and maintained low levels until the eighth day. On the tenth day, the levels were similar to those at the beginning of treatment but were significantly less than those of the control seedlings growing in the presence of N (Fig. 2B).

For Mayan Chan, this ratio decreased only on the eighth day. Additionally, when compared to the tenth day in both treatments this ratio was greater than that of the control seedlings growing in the presence of N (Fig. 2B). The SEMINIS variety reduced this ratio at the fourth day of treatment and remained at low levels at 8 and 10 days following treatment.

Table 1. The average value of the relative growth rate ($\bar{R}$) calculated for different species of habanero pepper growing in presence (N+) or absence (N-) during 10 days experiment. The data were calculated from aerial part dry weights mean values of three independent experiments. Different letters represent significant differences ($P \leq 0.05$) between varieties within the same treatment (N+ or N-) using Tukey's adjusted test for multiple comparisons.

Varieties	$\bar{R}$ (g DW/day)			
	Shoot		Root	
	N+	N-	N+	N-
Mayan Ek	0.14 ± 0.02a	0.11 ± 0.02a	0.14 ± 0.02a	0.09 ± 0.02a
Mayan Chan	0.07 ± 0.01c	0.04 ± 0.01b	0.06 ± 0.01b	0.05 ± 0.01b
SEMINIS	0.11 ± 0.02b	0.07 ± 0.01b	0.08 ± 0.02b	0.05 ± 0.01b

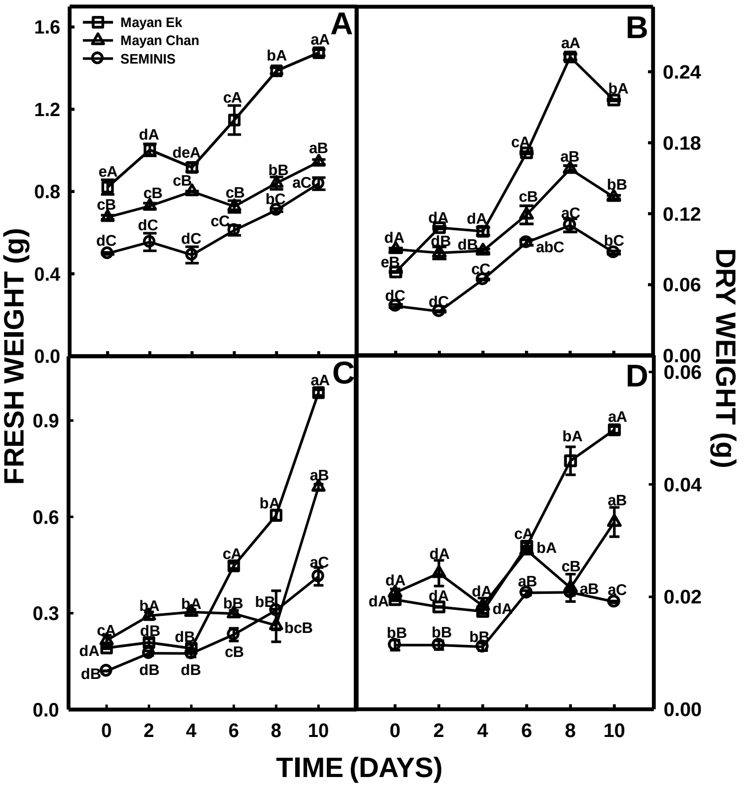

Fig 1. Growth response dynamics for three varieties of habanero pepper subjected to an N deficit for a period of 10 days, followed by the evolution of the fresh weights (A) and dry weights (B) of the shoots and fresh weights (C) and dry weights (D) of the roots. Figures show the data of a representative experiment of three repetitions. The data represent the mean value ± standard deviation (n = 3). The lowercase letters represent significant differences based on Tukey's test ($P \leq 0.05$) between the different days of N deficit within the same variety, and capital letters represent significant differences based on Tukey's test ($P \leq 0.05$) between varieties within the same day of treatment.

At the end of the experiment, this variety had a similar pattern to Mayan Chan, compared to the control, which was grown in the presence of N (Fig. 2B).

The seedlings shown in Figure 2C show the morphological changes described above for the 10-day seedlings subjected to the both treatments. Higher growth is observed in seedlings grown in the presence of N for 10 days, compared to those who grew under an N deficit. Mayan Ek presented a more developed root system and higher aerial part growth in comparison to the other two varieties and under all conditions (Fig. 2C).

The average relative growth rate values were consistently higher in Mayan Ek that in other varieties (Table 1). These values decreased in all varieties when they grew in the absence of N (Table 1).

Variability of nitrogen metabolites between habanero pepper varieties in response to N deficit

To explain the variations in habanero pepper growth rates during the deficit period and between varieties, metabolite levels associated with the assimilation of N were determined. The metabolites were nitrate, total amino acids and total protein in the roots and aerial parts of the plants. At the beginning of experiment, the nitrate values in the roots of all varieties were above those of the leaf, at 3.6, 6 and 9 times higher in varieties Mayan Ek, Mayan Chan and SEMINIS, respectively (Figs. 3A and 3B). Mayan Ek had the highest nitrate levels on that day, at nearly double those presented by the other varieties in both parts of the plant (Figs. 3A and 3B).

Table 2. Sequence of designed primer to amplify *CcNRT2.1* from habanero pepper roots.

Forward	Reverse
F1: 5´ ATG GCT GAT GTG GAA GGA GA 3´	R1: 5´ TCA GAC GCG GCT CGG TGT CA 3´
F2: 5´ GCT GCC GCC CCT TTA GTC CC 3´	R2: 5´ CGA AGA TCC ACG TCC TG 3´
F3: 5´ TCC TTT GTT TCG TCT GC 3´	R3: 5´ ACG CAA TTC TCC AAG CAG TG 3´
F4: 5´ ATA TTC GGC ATG AGA GGG AGA CTT T 3´	R4: 5´ TTG AGT TGC ACC GCC ACC CAT GTT T 3´
	R5: 5´ CGG CAG ACG AAA CAA AGG ACA TAC A 3´

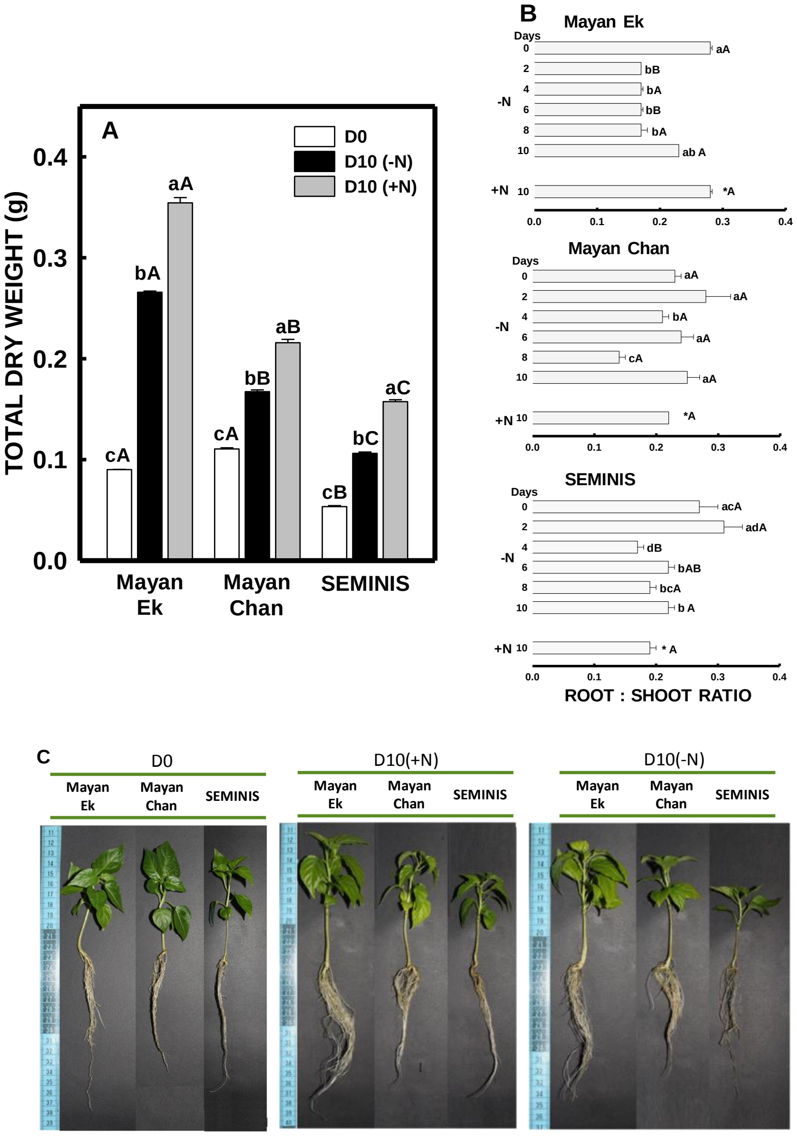

Fig 2. Growth response comparisons in habanero pepper varieties, subjected to a period of N deficit or in the presence of N (control). (A) Total dry weight of the seedlings at day 0 (D0) and day 10 growing under N deficit conditions [D10 (-N)] or in the presence of N [1 mM KNO_3, D10 (+ N)]. (B) Root dry weight/aerial part dry weight rate during the different days of N deficit and 10 days after growth in the presence of 1 mM KNO_3. Figures show the data of a representative experiment of three repetitions. The data in A and B are the mean value ± standard deviation (n = 3). The lowercase letters represent significant differences based on Tukey's test ($P \leq 0.05$) between the treatment within the same variety and the capital letters represent significant differences based on Tukey's test ($P \leq 0.05$) between varieties within the same day and/or treatment condition. Asterisk represents significant differences based on Tukey's test ($P \leq 0.05$) between plants within the same variety growing in the presence or absence of N by 10 days C. Seedling images for the three varieties of habanero pepper at the beginning of the experiment (D0) and 10 days after growing in the presence [D10 (+ N)] or deficit [D10 (-N)] of N.

The dynamics of this metabolite in the aerial parts during the deficit was similar for all varieties, with a similar increase when the deficit period increased, except for the Mayan Chan and SEMINIS varieties, in which the increase was less, or did not occur, after day 8 (Fig. 3A). The increased nitrate contents of the aerial part at the end of the deficit period was similar for the three varieties and corresponded to approximately 0.55 mg of nitrate per g of the aerial fresh weight (Fig. 3A).

However, the dynamics of root system nitrate in varieties exposed to a deficit was variety-dependent (Fig. 3B). Mayan Ek reduced the levels of this compound during all deficit periods, reaching a reduction of approximately 0.6 mg of nitrate per g of fresh weight (Fig. 3B). In contrast, the root nitrate levels increased in Mayan Chan during the first four days of the deficit and these levels subsequently declined, reaching lower values than those of Mayan Ek at the tenth day (Fig. 3B). At the end of the treatment period, there were no significant changes in the nitrate content in Mayan Chan relative to those that were present at the start of the experiment (Fig. 3B). The SEMINIS variety presented a similar behavior to Mayan Ek, because its nitrate levels decreased in seedlings roots exposed to the deficit. This decrease was equivalent to approximately 0.48 mg of nitrate per g of root fresh weight (Fig. 3B). However, the dynamic was not similar, and SEMINIS experienced reduced nitrate levels on day 2 of treatment, but subsequently increased and remained high until day 6, after which they began to decline again (Fig. 3B).

On the other hand, the amino acid levels were lower in the root relative to the aerial part at the beginning of treatment and in all varieties (Figs. 3C and 3D). Again, the Mayan Ek showed the highest values for this N metabolite in both plant parts.

The dynamics of the amino acid content during the deficit period was similar among varieties and in both parts of the plants A reduction in the content of these compounds was observed during this time (Figs. 3C and 3D). The reduction was almost linear in the roots of three varieties and the leaf of Mayan Chan. However, in Mayan Ek, a reduction in the amino acid leaf level was observed during the first two deficit days. The values were maintained until the day six and then subsequently decrease. For SEMINIS, the reduction continued until the 4th, and remained at a similar level until the sixth day and; thereafter, decreased, like Mayan Ek (Fig. 3C). The higher remobilization occurred in Mayan Ek leaf tissue, with approximately 2 mg of amino acids per g of fresh tissue (Fig. 3C). Mayan Ek maintained higher amino acid levels in both parts of the plant at the end of the period (Figs. 3C and 3D).

Although the total protein levels showed significant changes during the deficit (Figs. 3E and 3F), they were not as dramatic as those observed for nitrate and amino acids. Mayan Ek presented the highest protein values in the roots and leaves at the beginning of the experiment (Figs. 3E and 3F). Mayan Ek also had higher root protein level at the end of experiment, compared to the other two varieties. The leaf values were similar between Mayan Ek and Mayan Chan and were larger than those of SEMINIS.

Fig. 4 clearly shows the remobilization processes of nitrogenous metabolites mentioned above, with variations in their contents between the beginning and end of the deficit period. Fig. 4 also describes the contents of these metabolites in the leaves and roots of the control seedlings in the presence of 1 mM nitrate after growing for 10 days. When Mayan Ek was exposed to 1 mM nitrate, greater amino acid and protein levels were produced mainly in the aerial parts after 10 days of treatment, compared to other varieties (Fig. 4A), although this behavior was also noticed in the roots (Fig. 4B). This result confirms the greater assimilation of N that occurs in this variety under these conditions. To address the relationship between the total plant growth and the total contents of these metabolites, the control plants and those subjected to the deficit were studied for a period of 10 days. The results showed that the nitrate content increased with increasing plants dry weights (Fig. 5A). When plants were grown in the absence of N, there was a direct relationship between the concentration of this element and dry matter production. This relationship was independent of the plant variety. However, when grown in the presence of N, the effect of the nitrate content in the plant on dry matter production was dependent on the variety, and no Mayan Chan relation was observed under this condition (Fig. 5A).

The total amino acid content of plants had a direct impact on the dry weight; therefore, as these values increased in a linear fashion, the plant dry weight also increased (R^2 = 0.894, Fig. 5B), and this relationship was not dependent on the variety or the condition of N to which they were exposed. The relationship between the dry weight and total protein content was dependent on the condition of N to which they were exposed. For varieties under same condition, the relationship was not dependent on the variety (Fig. 5C). When the seedlings were grown in the presence of N, the impact of the protein content on the dry weight production was higher when compared with the N-deficient condition. The small changes in this parameter caused great changes in the seedling dry weight in comparison to values recorded in the absence of N (Fig. 5C).

Net nitrate absorption, characterization and evaluation of CcNRT2.1 transcript levels in habanero pepper varieties

An early plant response to a period of nitrate deficiency was the increase in high-affinity nitrate transport, which is determined primarily by AtNRT2.2 and AtNRT2.1 transporters in *Arabidopsis*. Based on differences between nitrogen metabolite levels of habanero pepper varieties, we evaluated the ability of different varieties to uptake nitrate under high affinity conditions when they were subject to a period of N deficiency, which was measured as the net absorption rate.

The net absorption rate increased in all varieties on the second day of the deficit, yielding the highest values for Mayan Chan, followed by SEMINIS and Mayan Ek, which subsequently decreased. It shows a second peak in all varieties on the tenth deficit day (Fig. 6A).

Surprisingly, negative values were obtained for the net absorption rate of Mayan Ek seedlings under six to eight days of deficit treatment (Fig. 6A). These values indicate that the nitrate efflux was higher than the influx during these times. To explore the molecular basis of this response, we studied the *CcNRT2.1* transcript levels in the three varieties. The *CcNRT2.1* is a total cDNA that was previously isolated from the roots of the SEMINIS variety, which shared a high portion of its identity with the nitrate transporter family of high affinity NRT2 type (data not shown). The transcript levels showed an increase in the expression following deficit treatment, which was related to variety (Fig. 6B). The *CcNRT2.1* transcript levels increased at days 2 and 4 and then

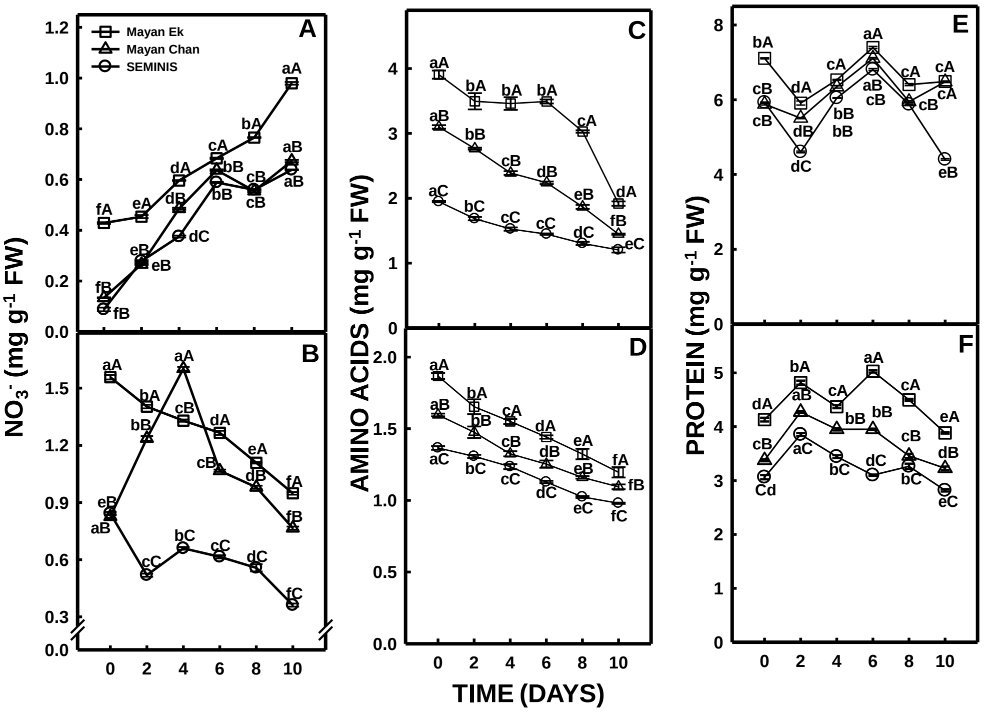

Fig 3. Nitrate levels (A and B), total amino acids (C and D) and total protein (E and F) in aerial parts (A, C and E) or root (B, D and F) of habanero pepper varieties during a 10 day N deficient period. Figures show the data of a representative experiment of three repetitions. The data represent the mean value ± standard deviation (n = 6). The lowercase letters represent significant differences based on Tukey's test ($P \leq 0.05$) between treatments within the same variety and capital letters represent significant differences based on Tukey's test ($P \leq 0.05$) between varieties within the same day of treatment.

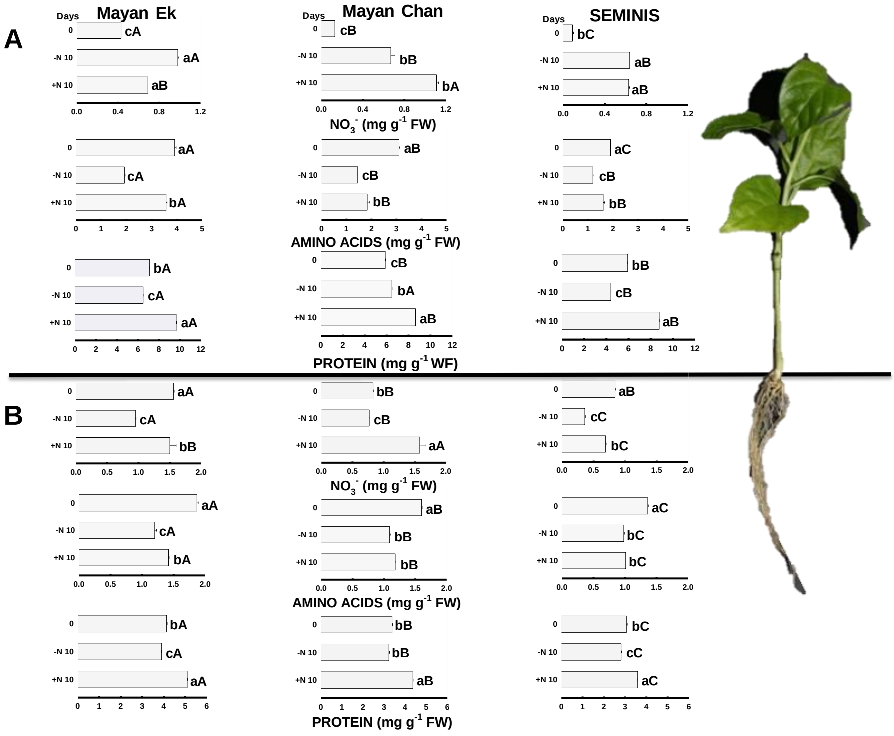

Fig 4. Comparison between control seedlings growing in the presence of N and seedlings exposed to N deficits for 10 days, with respect to the concentrations of nitrogen metabolites in roots (A) and aerial parts (B). Figures show the data of a representative experiment of three repetitions. Values represent the mean value ± standard deviation (n = 6). The lowercase letters represent significant differences based on Tukey's test ($P \leq 0.05$) between treatments within the same variety and capital letters represent significant differences based on Tukey's test ($P \leq 0.05$) between varieties within the same day of treatment.

disappeared in the Mayan Ek variety later (Fig. 6B). In contrast, the transcript values for Mayan Chan increased during the deficit, reaching higher values on the tenth day, similar to SEMINIS (Fig. 6B), but not completely coinciding with the net absorption values.

The PCR was performed to explore possible differences in expression levels of this transporter among varieties, using different combinations of primers, which were previously designed for the SEMINIS sequence. These combinations covered all the regions of this transporter (1593 bp long) and the predicted protein structure contained 12 transmembrane domains (Fig. 7). For this study, RNA from seedlings subjected to a deficit period during day 2 was selected from the Mayan Ek and Mayan Chan varieties, and the sequence from SEMINIS was previously cloned. As shown in Fig. 7, the expected fragment was not amplified at the all times, and this happened to Mayan Chan with primer sets F2-R3 and to Mayan Ek with the F3-R2 primer set. It was not possible to amplify the cDNA with primer set F1-R1 (Fig. 7).

In summary, there were significant response differences between the varieties in the absence treatment of N. The Mayan Ek variety presented the highest fresh weight and dry weight values at the end of the deficit period and had a more developed root system (e.g., the shoot). All evaluated nitrogenous metabolites were also higher in this variety when compared with Mayan Chan and SEMINIS. Mayan Ek showed higher remobilization of root nitrate and amino acids in both parts when exposed to the treatment. In contrast, the Mayan Chan variety showed a greater high affinity nitrate uptake. All varieties increased their *CcNRT2.1* transcript levels under an absence of N, but in different manners.

Discussion

Effect of an N deficiency period on the growth variables of three habanero pepper varieties

Growth data on fresh and dry weight indicated that the Mayan Ek variety was less susceptible to N deficit than other two varieties. This ability to grow better in the absence of an external source of N shows that Mayan Ek can remobilize reserve endogenous N more efficiently than SEMINIS and Mayan Chan. Growth parameter differences among cultivars of same species, when plants are subjected to limiting conditions or N deficiency, have been previously reported (Richard-Molard et al., 2008; Shi et al., 2010). In hydroponic conditions, two rice cultivars had increased dry shoot and root weights when subjected to limiting concentrations of N (Shi et al., 2010). However, one managed to achieve higher dry matter during the same period, proving to be a variety that had introduced greater NUE under field conditions (Shi et al., 2010). In *Arabidopsis*, there was also an increase in the fresh and dry weights of both plant parts when grown under an N deficit (Krapp et al., 2011). The decrease in the root/aerial dry weight ratio in variety Mayan Ek did not coincide with those previously reported for grasses (Jarvis and Macduff, 1989), tea (Anandacoomaraswamy et al., 2002) and *Arabidopsis* (Richard-Molard et al., 2008; Krapp et al., 2011), which experienced an increase in the ratio during N deficit condition. These authors suggested that a carbon deviation occurs in the underground portion which maintain or stimulate root elongation. In our case, the Mayan Ek variety kept a constant root dry weight during the first four days of treatment deficit. However, the aerial part increased during this period, leading to an observed reduction for this ratio during those days. These results demonstrated various adaptive responses between different varieties in terms of dry matter partitioning, in which Mayan Ek showed a faster impact under deficit N to change this parameter from the two-day deficit.

Mayan Ek presented further root system development. The more developed root system could maintain higher growth in the aerial parts and optimize the acquisition of soil N when there are decreased concentrations of this element. In barley, genotypes identified to be relatively efficient for N use in the field had a higher root dry weight than others (Anbessa and Juskiw, 2012). Furthermore, a biomass increase has been implicated as the major physiological basis of genetic gain in crops yields, which is an important component of NUE (Tollenaar et al., 2004). The high biomass production presented by Mayan Ek must be the result of the greater use of previously absorbed N when compared to the other varieties.

The decreased average relative rates of growth are indicative of the fact that the resources required for growth are not proportional to the growth rate of the plant. It has been proposed that the value differences between species may be important in natural ecosystems and in fast growing varieties, such as Mayan Ek, the dominant variety in disturbed habitats or those with high productive potential (Gregory, 1992).

Effect of an N deficiency period on nitrate, amino acids, and protein contents in three varieties of habanero pepper

Higher nitrate contents at the beginning of the experiment showed that the Mayan Ek variety might be capable of greater nitrate absorption and/or tissue storage when exposed to normal nitrate growing condition, compared to the other two varieties. Moreover, the greatest reduction in root nitrate levels during the deficit period suggests that this variety could remobilize the stored nitrate from the root to the aerial parts to maintain normal growth under N deficit conditions and/or increase assimilation in the plant root.

A reduction in nitrate levels has been reported both in the aerial portion as well as in the root system of various plant species, when subjected to a period of nitrate deficit. This decrease was more rapid than that of other nitrogen compounds (van der Leij et al., 1998; Richard-Molard et al., 2008; Ding et al., 2011; Krapp et al., 2011). The results obtained here for Mayan Ek and SEMINIS root systems agree with those of previously reported works. This behavior is evidence that habanero pepper plants mobilize their reserves to maintain N metabolism in the early days of N deficiencies, particularly Mayan Ek. However, this does not occur in the aerial part. The initial nitrate values in habanero pepper show that this species accumulates more in the root when exposed to nitrate, suggesting a lower nitrate assimilation in the root and/or increased assimilation in the leaves and/or a lower translocation of this compound from the roots to the leaves. Because this nutrient is accumulated mainly in the roots of this species, when grown in the presence of N, it is likely that this factor (not that of the aerial part) is the pool of nitrate that remobilizes under deficit conditions; and therefore, this might be a mechanism-dependent variety.

The *Arabidopsis* accumulated three times more nitrate in the aerial parts than in the root after 35 days in the presence of 6

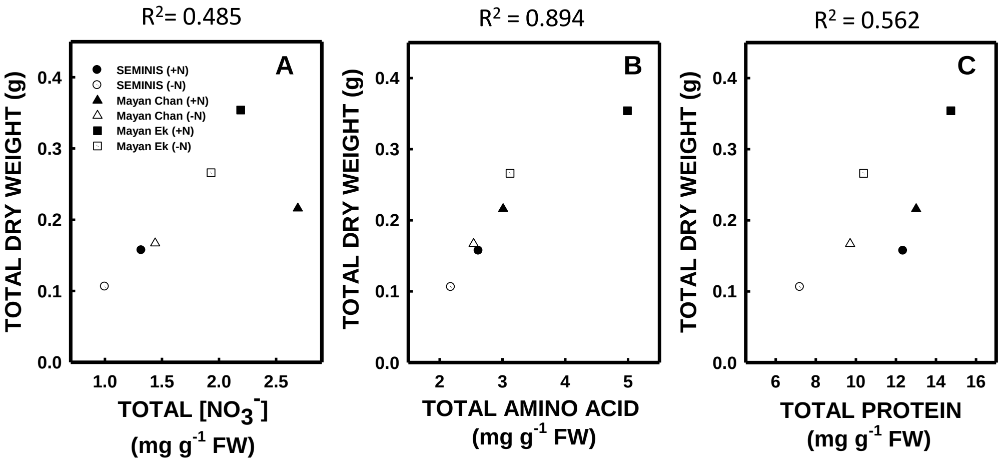

Fig 5. Relationship between the growth of habanero pepper seedlings and the (A) total values for nitrate, (B) total amino acids and (C) total protein. These graphs were used to find the sum of the mean values of each determination in both roots and aerial parts.

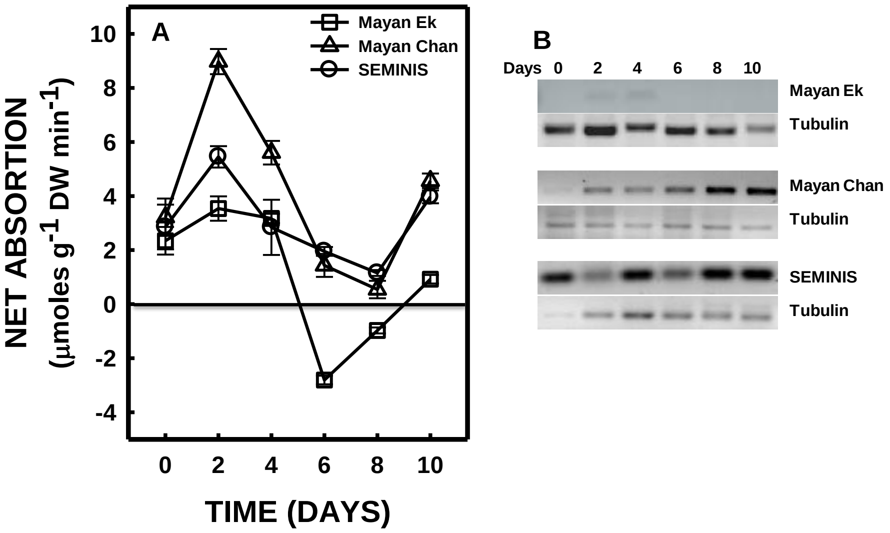

Fig 6. Characterization of net absorption capacity and *CcNRT2.1* transcript levels of habanero pepper varieties subject to nitrogen deficiency. (A) Net nitrate absorption rate. For this measurement, habanero pepper seedlings of each variety were collected following 0, 2, 4, 6, 8 and 10 days of N deficit and placed (six seedlings per treatment) in a solution containing 0.2 mM nitrate. The concentration of this element was measured before and after incubation to calculate the net absorption rate as indicated in materials and methods. Figures show the data of a representative experiment of three repetitions. The data represent the mean value and the error bar indicates the mean standard deviation (n = 6). (B) *CcNRT*2.1 transcript levels using the combination of primers F4-R1. The tubulin gene was used as a positive control.

mM nitrate, but the nitrate levels were reduced by 70%, both in the aerial part and in the root during the first 24 hours (Krapp et al., 2011). A greater accumulation of nitrate in the leaves also seems to be a general feature of cereals (Fan et al., 2007).

Nitrate is considered not only as a nutrient source but also reported to act as a signal within plants. Nitrate regulates the expression of 300 to 600 genes in a specific manner and with a certain number of minutes; therefore, it belongs to different functional categories, such as ion transport, primary and secondary metabolism, hormone homeostasis, transcription and RNA processing, among others (Wang et al., 2004; Krouk et al., 2010b). Nitrate also stimulates the number of lateral roots (Zhang et al., 1999; Krouk et al., 2010a) and root elongation (Zhang et al., 1999; Remans et al., 2006). Moreover, the growth of the aerial part is equally depends on nitrate signaling, in which it regulates the expansion of the leaf through the stimulation of cytokinin biosynthesis in the root and cytokinin translocation to the aerial portions (Kiba et al., 2011). The signaling role of nitrate should not be ruled out in this work, given the variation in endogenous concentrations of this element between varieties with different growth rates.

The amino acid levels in both parts of the plant confirm that habanero pepper has higher N assimilation in the aerial part than in the root. The Mayan Ek values showed higher value of N metabolite in both plant parts, indicating that this variety must have greater N assimilation when grown in the presence of nitrate in comparison to the other two.

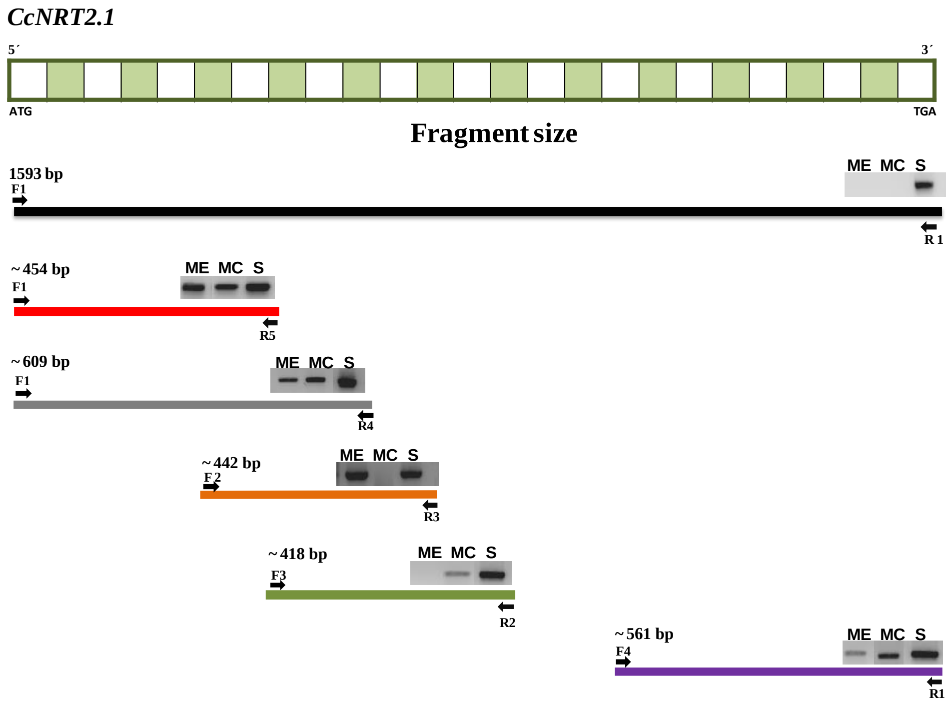

Fig 7. Differences in *CcNRT2.1* sequences between habanero pepper varieties. The diagram shows the structure of this transporter with 12 predicted transmembrane domains (gray) and the different fragments that were amplified with their respective sizes. The right end of each fragment includes the PCR result obtained in each case. ME: Mayan Ek, MC: Mayan Chan, S: SEMINIS.

The minimum amino acid pool was higher in Mayan Ek, and it could be vital for the synthesis of essential proteins or other compounds with higher turnover rates under these conditions. Our results agree with those reported for *Arabidopsis* (Richard-Molard et al., 2008; Krapp et al., 2011) and tobacco (Fritz et al., 2006). Recently, it was reported that the amino acid content of the roots was a regulatory trait that coordinated *Arabidopsis* growth when the plant is subject to limitated N (Ikram et al., 2012). Minor protein modifications occurred during the N deficit treatment relative to nitrate. The amino acids identified in this study have also been previously reported in *Arabidopsis* (Richard-Molard et al., 2008). The protein content is an important consideration. Wheat cultivars with high protein concentrations were more efficient in translocating N from vegetative tissue to the grain (Wang et al., 2003; Kade et al., 2005). Wang et al., (2009) reported that a wheat cultivar with high protein concentrations in the vegetative tissue had a higher harvest index (percentage of dry matter diverted to grain) than those with a lower concentration. Suprayogi et al. (2011) also reported that the protein concentration in the grain of different wheat varieties was higher in those with a higher protein concentration. There was a positive correlation between the total amino acid content and the amount of dry matter in the plant, suggesting that habanero pepper varieties with greater total amino acid contents may have a greater total dry weight accumulation under any given conditions. Therefore, this parameter could be an important marker for dry matter accumulation in this species.

Effect of N deficiency period on the high affinity nitrate uptake and the transcript levels of high affinity nitrate transporter CcNRT2.1 gene in three habanero pepper varieties

The high-affinity nitrate uptake detected in habanero pepper varieties during short treatment times coincided with what happens in other plants such as rice (Shi et al., 2010) and *Arabidopsis* (Richard-Molard et al., 2008), but not for long-term trends. This second increase in high-affinity nitrate uptake during periods of N deficiency might indicate a new regulatory mechanism, which is apparently common in habanero pepper. This behavior was similar for all varieties. Although a negative net absorption of nitrate was already reported for other plants under stressed conditions (Macduff and Jacksson, 1992; Aslam et al., 1996; Dehlon et al., 1995), the biological significance of this response is unknown. A transporter, responsible for nitrate efflux, has been characterized in *Arabidopsis*. It belongs to a subclass of the seven-member NAXT (excretion NITRATE TRANSPORTER), but this family is not known within the genus *Capsicum* yet. This result is novel and cannot explain the efflux in Mayan Ek under relatively long period of N deficiency (six days). It would be interesting to study the different nitrate transporters in the roots of this variety under the conditions of this study. One possible explanation could be that an efflux transporter is responsible for activating nitrate in habanero pepper roots, which results from an increase in the concentration of this ion in the cytosol.

This increase could occur as a result of remobilization following deficit treatment, during which nitrate is removed from the vacuole for assimilation in the cytosol. The remobilization of nitrate from the vacuole into the cytosol under deficient conditions has been previously reported (Wang et al., 2012). Mayan Ek has greater nitrate values in the roots and greater remobilization during this deficit, as described above; therefore, reaches higher cytosol values relative to the other varieties. This would explain why no negative values were obtained in the other species, despite a reduction in the net absorption rate during this period. Nitrate efflux has been proposed previously as a mechanism for regulating the homeostasis of this ion under fluctuating external nitrate conditions (Huang et al., 2012). A transient increase in the transcript levels of high affinity nitrate transporters when plants are exposed to this limiting conditions has been previously reported (Richard-Molard et al., 2008; Shi et al., 2010). Similarly, a poor correlation between the transporter transcript levels and its activity has been reported (Kaiser et al., 2002). It is also known that the *Arabidopsis* NRT2.1 transporter can be regulated at the post-translational level (Wirth et al., 2007; Barbier-Brygoo et al., 2011). Other carriers may also contribute to the high affinity uptake of nitrate in this species. The results obtained here suggest that even when Mayan Ek stimulates high affinity uptake when grown under deficit conditions, this uptake does not seem to be the major mechanism for this variety. In this case, Mayan Ek seems to prefer the remobilization of endogenous reserves. However, because endogenous N reserves are lower in the Mayan Chan variety, this variety seems to favor the activation of high affinity nitrate uptake as an adaptive response to conditions of N deficiency.

The two Yucatan variety transporters had different genic regions of the SEMINIS variety and also between these two Yucatan varieties. These differences seem to be concentrated in the central region of this transporter gene. It would be interesting to study the regulation of nitrate uptake in these varieties, given the variations observed in this study, and the role that transporters in this process.

Materials and Methods

Plant material, growth conditions, and experimental treatment

In this study, we used three varieties of habanero pepper; one was an orange cultivar marketed by SEMINIS (SEMINIS Vegetable Seeds, Inc. 2700 Camino del Sol, Oxnard, CA 93030, USA), which is commonly grown in Mexico and other parts of the world, and the two other varieties were collected in different parts of Yucatan, Mexico, and belong to Dr. Nancy Santana Buzzy collection of habanero peppers (Scientific Research Center of Yucatan). The Mayan Ek® variety (National System of Seed Inspection and Certification, SNIC, register number: 2370-chl-024-080110/c) is an orange habanero pepper that is highly pungent, and the Mayan Chan® variety (National System of Seed Inspection and Certification, SNIC, register number: 2369-chl-023-080110/c) is red. The varieties of Yucatan region were selected by their higher productivity and fruit quality (highly pungent).

Seeds were surface sterilized using 80% ethanol (v/v) for five minutes. After four washes with sterile distilled H_2O, the seeds were placed in sodium hypochlorite [30% (v/v), CloralexTM, North Alen, SA de CV] for 15 minutes and again washed four times with distilled H_2O. These seeds were subjected to a stratification process in water at 4 °C for three days. The seeds were placed in glass Petri dishes (50 seeds per dish) containing a thin layer of cotton, sterile filter paper and 10 mL of distilled H_2O and kept in a dark room at 24 ± 2 °C until germination (about the fifth day of incubation). After germination, the seeds were placed in 400 mL containers with sterile vermiculite (70 g of vermiculite and 15 seeds per pot) and the seedlings were grown for 45 days in a growth room at 25 ± 2 °C, a relative humidity of 50% and a photoperiod cycle of 16 h light/8 h dark. The light intensity was 123 µmol m^{-2} s^{-1}. Modified Hoagland nutrient solution was used (50 mL per container) to 1/5 of its ionic strength, and contained 0.1 mM CaCl, 2.5 µM H_3BO_3, 0.2 µM $MnSO_4$. H_2O, 0.2 µM $ZnSO_4$, 0.04 mM $CuSO_4$. 5 H_2O, 0.02 µM Na_2MoO_4.2H_2O, 2 µM Fe-EDTA, 0.04 mM KH_2PO_4, 0.04 mM $MgSO_4$ and 1 mM KNO_3 as a unique N source and the pH was adjusted to 5.8. The nutrient solution was replaced by a fresh solution once during the first fifteen days of growth and then every week until 45 days had elapsed. The vermiculite was maintained in a fully moist state by adding sterile distilled H_2O (approximately 50 mL) on the fourth day after each medium renewal to avoid salt accumulation by water evaporation. The vermiculite was subsequently removed and the plantlets were transferred for acclimation to hydroponic conditions after four days in the medium described above, but to ½ of its ionic strength, and kept in 1 mM KNO_3.

To treat the N deficit, a group of seedlings from each variety were transferred to a nutrient solution (0.5 mM CaCl, 12.5 µM H_3BO_3, 1 µM $MnSO_4$. H_2O, 1 µM $ZnSO_4$, 0.5 µM $CuSO_4$. 5 H_2O, 0.1 µM Na_2MoO_4.2H_2O, 10 µM of Fe-EDTA, 0.2 mM KH_2PO_4, 0.2 mM $MgSO_4$), and KNO_3 was removed from the medium and 1 mM KCl was added to maintain the K levels. Harvests were carried out at 0, 2, 4, 6, 8 and 10 days of deficit treatment and the plant material was quickly frozen in liquid N, and stored at -80 °C for subsequent molecular and metabolite level analyses. Twenty seedlings were used for each harvest (180 total seedlings).

Another group of seedlings was transferred to the nutrient solution described above to be used as a control, but the KNO_3 treatment was maintained at 1 mM. The harvest of this material was performed only after 10 days of exposure, and the plants were treated in the same manner as outlined above. Three independent experiments were performed under the same conditions.

Fresh and dry weight measurements and determination of the average relative growth rate

Seedlings subjected to the above treatments were photographed (Canon EOS Rebel Tli) at each harvest time (three seedlings per treatment). The roots of these seedlings were dried with paper towels and separated from the shoots immediately after harvest and then weighed to obtain the fresh weights of both parts. To determine the dry weight, the material was placed in the oven at 70 °C until reaching a constant weight.

The average value of the relative growth rate ($\bar{R}$) was calculated according to the formula reported by Gregory, (1992).

Nitrate, amino acid and total protein analyses

For the nitrate analysis, 100 mg of leaf and root were used and the extraction and determination (in mg g^{-1} fresh weight) were performed following the methodology described by Cawse, (1967), using a standard KNO_3 curve (Sigma-Aldrich).

For the amino acid analysis, 200 mg of roots and leaves were pulverized with liquid N. A 1 mL of 80% methanol (v/v) was added and the extract was centrifuged at 10 000 xg for 20 minutes. The supernatant was used to determine the total amino acids (mg g^{-1} fresh weight) following the methodology of Yemm and Cocking, (1955).

For the protein extraction, approximately 1 g of fresh roots and leaves was macerated in liquid N, followed by the addition of 1% polyvinylpoly pyrrolidone (w/v), the extraction buffer (2.5 mL, 50 mM NaCl, 1 mM EGTA, 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 10% glycerol, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 10 mM

$Na_4P_2O_7$, 0.2 mM Na_3VO_4, and 1 mM β-mercaptoethanol) was added and homogenized in a polytron. The supernatant collected following centrifugation at 12 000 xg for 30 minutes at 4 °C was used for the total protein determination (mg g^{-1} fresh weight), by Bradford method (1976), following the manufacturer's protocol (BioRad, Cat 500-0006). There were two different extracts and all the determinations were performed in triplicate assays.

Net nitrate absorption assay

To test the net nitrate absorption, seedlings exposed to an N deficit. They were placed in a solution containing 0.2 mM KNO_3 for 15 minutes. The nitrate content in the medium was measured before and after incubation with this nutrient, following the protocol described above and by using 1.5 mL of the previously filtered medium. The net absorption rate of NO_3^- (µmol g^{-1} root dry weight $minute^{-1}$) was calculated from the difference between the initial and final content of NO_3^- in the medium. The root dry weight was determined as described above. In this test, six seedlings were used per treatment.

Analysis of transcript levels of CcNRT2.1 gene by RT-PCR

To evaluate the expression levels of high nitrate affinity transporter *CcNRT2.1* gene, habanero pepper seedlings were subjected to different periods of N deficiency. Total RNA was isolated from the root tissue of these seedlings with TRIZOL reagent (Invitrogen). The cDNA synthesis was performed in all cases starting from 1 µg of total RNA and using the enzyme reverse transcriptase SuperScripTM (Invitrogen) with oligo-dT, as described in the protocol provided by the manufacturer. For PCR, we used primers F4 and R1 (Table 2), which were previously designed from the total cDNA sequence from *CcNRT2.1* isolated in the laboratory from roots of SEMINIS variety habanero pepper. We started with 1 µl of cDNA (~ 100 ng) and used Platinum *Taq* polymerase (Invitrogen). The PCR conditions were as follows: 96 °C for 2 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 40 s, and 72 °C for 10 min. The amplification product was separated and visualized on a 1% agarose gel stained with ethidium bromide. Tubulin served as a positive control in the reaction with primers: F, 5 'GACCTTGAATCGGCTTATGG 3' and R-5 'TATCCTG-GGTGAACGCTTTG 3'. For this study, RT-PCR was performed with two different RNA extracts from each treatment and PCR was performed in duplicate for each sample.

To investigate the characteristics of this transporter in Mayan Chan and Mayan Ek varieties, several primers were designed to amplify different transporter regions. In this case, the RNA samples from two days of nitrate deficit were selected from Mayan Chan and Mayan Ek. PCR was performed using the following primer combinations (Table 2): F1-R5, F1-R4, F2-R3, F3-R2 and F1-R1, and the expected sizes of these PCR products were 454, 609, 442, 418 and 1593 bp, respectively. The PCR conditions were similar to those mentioned above, with only the times of polymerase reactions changed according to the size of the expected product, and on this occasion the number of cycles was 40.

Statistical analysis

All data were subjected to a two-way analysis of variance (ANOVA) and differences between the means were compared by Tukey's multiple range test ($P \leq 0.05$) using SIGMA STAT statistical package v.11.

Conclusion

The results shown here indicate that the Mayan Ek variety seems to make more efficient use of N when grown in the absence of this element because it was able to produce drier matter under the same deficit conditions as others varieties. The Mayan Ek variety possess a high capacity to store nitrate and assimilation products (amino acids and proteins) prior to the deficit period and a high capacity for nitrate remobilization and amino acids when absent. Mayan Chan was the second best choice to tolerate the deficit period and was also evaluated for dry matter production. This variety appears to stimulate high affinity nitrate uptake and to induce the transcription of *CcNRT2.1* transporter for long periods of deficit. This is the first work in habanero pepper that evaluates changes in growth under nitrate deficiency using morphological, physiological and molecular traits, and it illustrates the variability in responses between different varieties of this species. This work lays the foundation for future breeding programs, with the aim of obtaining and growing plants that make more efficient use of N under nutrient-limiting conditions, such as those found in the soils of Yucatan.

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