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Revealing molecular adaptive response in maize (*Zea mays* L.) under nitrogen starvation stress in low fertile sandy soil

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ABSTRACT

Developing maize genotypes with better resilience to nitrogen (N) starvation is a promising sustainable strategy for minimizing both nitrogen fertilizers cost and nitrogen pollution in water and air within the Egyptian agroecosystem. In this work, we examined 12 different Egyptian maize hybrids in terms of phenotypical response to prolonged nitrogen starvation. Subsequently, 4 maize hybrids were selected to represent more-sensitive (TWC 360 and TWC 321) and less-sensitive (SC 128 and SC 130) responses to severe N deficiency in soil for further phenotypical, biochemical, and molecular examination. The applied nitrogen (N) starvation, achieved by completely preventing N fertilization in sandy soil for three months, significantly reduced both shoot dry weight (SDW) and root dry weight (RDW). SC 130 exhibited the lowest reduction ratio at 71% and 54%, respectively. Regarding root traits, the cross-section area (CSA) was dramatically diminished due to N starvation, with a reduction ratio of 64% and 61% in TWC 360 and TWC 321 (more sensitive), and 50% and 33% in SC 128 and SC 130 (less sensitive), respectively. The applied nitrogen starvation stress significantly reduced total chlorophyll content, with SC 130 showing the highest level (2.05 mg chlorophyll/g FW) compared to 0.97 mg chlorophyll/g FW in TWC 360. For the quantitative gene expression profile of several nitrogen stress marker genes, catalase, basic endochitinase, and nitrate transferase one genes were significantly upregulated in the less sensitive hybrids SC 128 and SC 130 leaves. On the other hand, the high-affinity transporter gene 2.3 was significantly reduced in the less sensitive hybrids TWC 360 and TWC 321.

Keywords: Maize; *Zea mays* L.; nitrogen starvation; gene expression; root traits

INTRODUCTION

Nitrogen (N) is a fundamental macronutrient essential for optimal plant growth and development; it is a primary component of amino acids, chlorophyll, adenosine triphosphate (ATP), and nucleic acids (DNA and RNA) (Wang *et al.,* 2017; Ostria-Gallardo *et al.,* 2024). However, nitrogen is the most expensive plant nutrient applied to cultivated crops to increase agricultural productivity and economic profits (Urban *et al.,* 2021). Extensive or prolonged applications of nitrogen fertilizers result in elevated crop production costs and environmental contamination (Anas *et al.,* 2020). Xia and Yan (2023) reported that only about 40% of nitrogen added to soil for agricultural production is absorbed by plants (i.e., nitrogen use efficiency or NUE is 40%). In contrast, more than half of the nitrogen leaks into the environment as gaseous emissions and leached dissolved nitrogen into water bodies, particularly groundwater (Zhang *et al.,* 2015; Liu *et al.,* 2024).

Maize (*Zea mays* L.) is the third most economically strategic cereal crop in Egypt, after wheat and rice, with a production value of 750,000 tons from a cultivation area of 930,000 hectares (2.23 million feddans) and a production rate of 8 tons per hectare (FAO, 2022). Maize, or corn, is an extremely versatile cereal crop with a significant demand for nitrogen (N) fertilization (Sheoran *et al.,* 2021). Several approaches exist for modifying nitrogen fertilization to minimize environmental contamination while maintaining optimal yield levels. These approaches include adjusting the right fertilizer type, amount, placement, and timing (Gu *et al.,* 2023) or modifying soil texture by adding amendments such as biochar or compost (Chi *et al.,* 2024). Nevertheless, developing maize hybrids with considerable adaptation to nitrogen deficiency in soil, or in other words, better nitrogen use efficiency (NUE), would greatly support and complement such efforts (Singh *et al.,* 2022).

However, this task requires a comprehensive understanding of the molecular response of maize to severe low nitrogen deficiency. This work was conducted to phenotype several Egyptian maize hybrids in response to nitrogen starvation stress and quantify the gene expression profile of key genes associated with nitrogen metabolism (uptake, assimilation, and reallocation) in four Egyptian maize hybrids with variable responses to prolonged nitrogen starvation stress in low-fertility sandy soil, exemplifying the newly reclaimed sandy soil regions in the Egyptian agroecosystem.

MATERIAL AND METHODS

Plant materials and N-stress treatment:

Twelve Egyptian maize hybrids were provided by the Field Crop Research Institute (FCRI), Agricultural Research Center (ARC), Giza, Egypt. The phenotypically screened maize hybrids are TWC 360, TWC 368, TWC 321, TWC 352, TWC 324, SC 173, SC 131, SC 168, SC 176, SC 128, SC 132, and SC 130. The Egyptian maize hybrids were germinated in two types of sandy soil (50% sand, 25% top soil, and 25% compost): one representing normal nitrogen (NN) with optimal doses of nitrogen fertilization (8 g of urea per plant), and the second representing nitrogen starvation stress (NS) where no nitrogen fertilizers were added until harvest after 3 months. The soils were periodically supplied with other macronutrients such as phosphorus, potassium, sulfur, and calcium, along with a mixture of micronutrients like boron and zinc to prevent any unplanned nutrient deficiency symptoms. The rate of fertilization was performed according to the guidelines of the Agricultural Extension, Ministry of Agriculture and Land Reclamation (MALR), Egypt.

The plants were allowed to grow until the age of 3 months (late vegetative stage), and then they were photographed and non-parametrically divided into two groups based on the phenotype of stressed plants, i.e., exogenous appearance. Four hybrids were further selected to represent the most sensitive hybrids to nitrogen starvation stress (TWC 360 and TWC 321) and the least sensitive hybrids (SC 128 and SC 130). Shoots were harvested using sharp pruning scissors, and peripheral parts were collected, frozen instantly in liquid nitrogen (LN), and stored at -80 ℃ for subsequent biochemical and molecular analysis. Root systems are extracted and delicately washed using a water stream from a hose until all soil debris is completely removed. They are then stored in 70% ethanol for subsequent anatomical and architectural trait investigations.

Plant measurements:

Shoot growth measurement:

At harvest, following a three-month episode of nitrogen starvation stress, the shoot system of all treated plants was separated from the root system using sharp and clean garden scissors. The shoots were then air-dried for two weeks and subsequently dried in an oven at 80 ℃ for three days before being weighed to determine dry biomass. The percentage reduction of shoot dry biomass in NS maize plants was calculated using the following equation: [(NN–NS)/NN] * 100, where NN is the weight of shoots from the normal nitrogen control treatment, and NS is the weight of shoots from nitrogen-starvation-stressed plants.

Root growth measurement and anatomical traits:

The root systems of treated maize plants were extracted from the soil in the greenhouse using a high-pressure water stream and then delicately washed in the laboratory several times with tap water to remove any possible traces of soil granules. They were then stored at 4 ℃ in 70% ethanol for subsequent root trait investigations. For studying anatomical traits, root segments 2 cm from the basal position were hand-sectioned using sharp mid-thickness blades, stained with 1.5% Toluidine Blue O for 5 minutes, and then washed 2–3 times with distilled water. The transverse sections were examined and imaged using the stereoscope SZ61 (Olympus, Japan) equipped with a high-resolution digital camera DP23 (Olympus, Japan). Using the software ImageJ, the cross-section area and stele area were measured. The ethanol-preserved root samples were briefly rinsed with distilled water and then dried in an oven at 80 °C for two days to record root dry biomass. The root biomass reduction ratio was calculated for each sample as follows: (NN–NS/NN) * 100. NN: weight of shoots of normal nitrogen control treatment, NS: weight of shoots of nitrogen starvation-stressed plants.

Chlorophyll content:

Total chlorophyll was estimated in the leaves of the four examined maize hybrids under normal nitrogen control and nitrogen starvation stress, according to (Rajalakshmi and Banu, 2015).

Molecular analysis:

Total RNA was isolated from the leaves of the four examined maize hybrids under normal nitrogen control and nitrogen starvation stress using Direct-zol RNA Miniprep (Zymo Research, USA). cDNA was synthesized using a commercial kit (Willowfort, England) following the supplied protocol. Quantification of gene expression profiles for several stress marker genes was achieved through conventional PCR via the Gel express method (Hazman, 2022). Six genes related to nitrogen uptake, assimilation, and reallocation were quantified. Primer sequences used in this work can be retrieved from (Singh *et al.*, 2022).

Statistical analysis:

The completely randomized design was applied to experiments. Means were compared using multiple range tests according to (Duncan, 1955) using computer program SPSS, IBM, USA.

RESULTS

phenotyping wide range of Egyptian maize hybrids in response to N starvation:

Twelve Egyptian maize hybrids were screened by applying nitrogen starvation stress, with no nitrogen fertilizers added throughout a 3-month growth period until the late vegetative stage, just before inflorescence, under greenhouse conditions. The examination of stressed shoot systems showed that nitrogen starvation stress severely diminished plant height (Supp. 1) and caused nitrogen deficiency symptoms, such as yellowish V-shaped leaf tips.

The noted reduction in plant height and the appearance of nitrogen deficiency symptoms confirmed the effectiveness of applied nitrogen stress in triggering these symptoms, revealing possible variations among the examined maize hybrids. Based on this exogenous non-parametric classification, it was possible to select four different maize hybrids: SC 128 and SC 130 (less sensitive) and TWC 360 and TWC 321 (more sensitive). There was a phenotypic difference between the response of hybrid TWC 321, as a more sensitive maize hybrid to nitrogen stress, and hybrid SC 130, as less sensitive. SC 130 shoots appeared to be higher than TWC 321 under nitrogen-starvation growth conditions (Fig. 1).

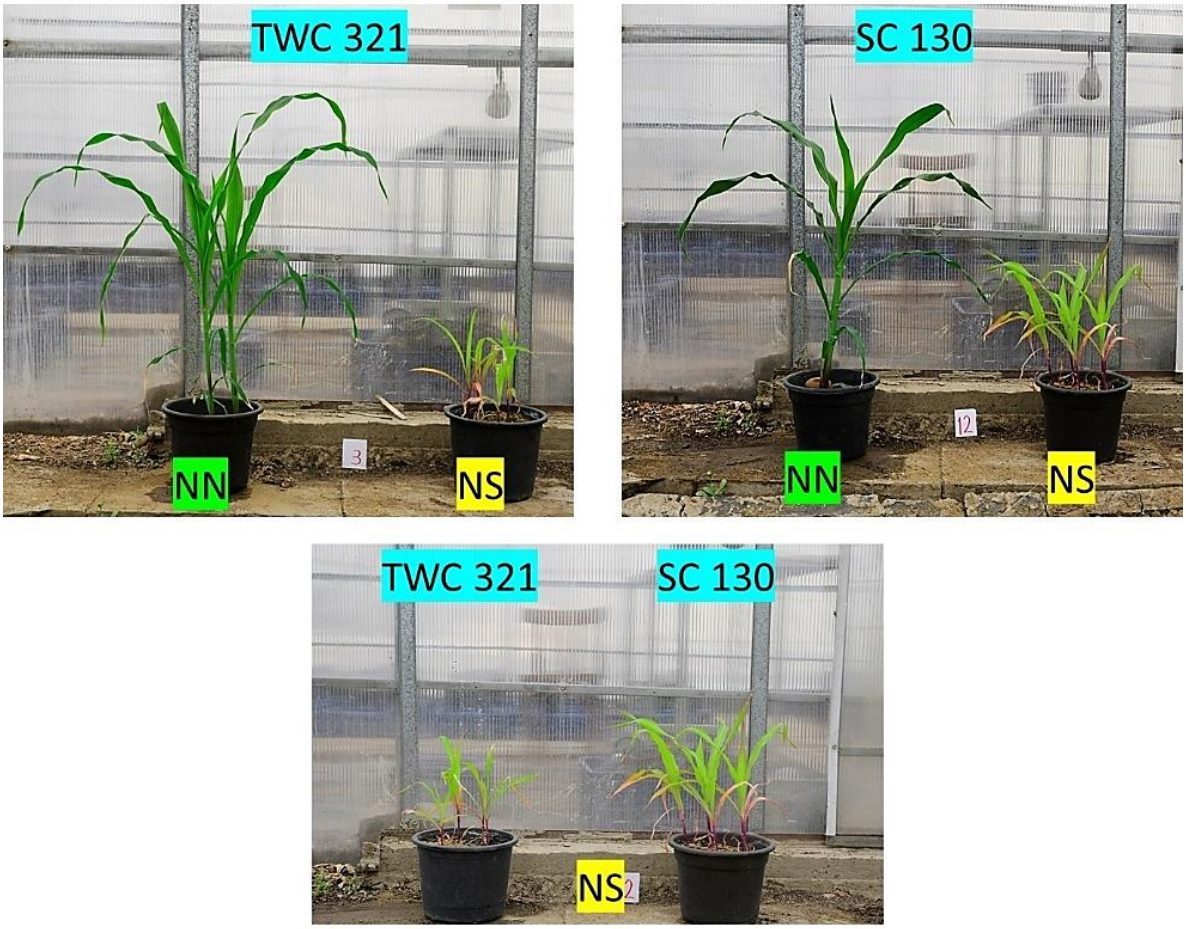

Fig. 1. The phenotypic differences between the more-sensitive maize hybrid TWC 321 and the less-sensitive hybrid SC 130 in response to N starvation stress.

3.2 Shoot dry weight and root dry weight of N stressed maize hybrids:

Applied nitrogen starvation stress significantly inhibited the shoot dry weight (SDW) of the four selected hybrids. Nevertheless, the more sensitive hybrids, TWC 360 and TWC 321, showed the highest reduction in shoot dry biomass (82% and 86%) compared to the less sensitive hybrids, SC 128 and SC 130, which showed the lowest reduction ratios of 72% and 71%, respectively (Fig. 2A). For root dry weight (RDW), nitrogen starvation stress also strongly reduced values in all four maize hybrids. The root dry weight of maize hybrids TWC 360 and TWC 321 (more sensitive) showed the largest reduction ratios (74% and 70%) compared to SC 128 and SC 130 (less sensitive), which were 59% and 54%, respectively (Fig. 2B).

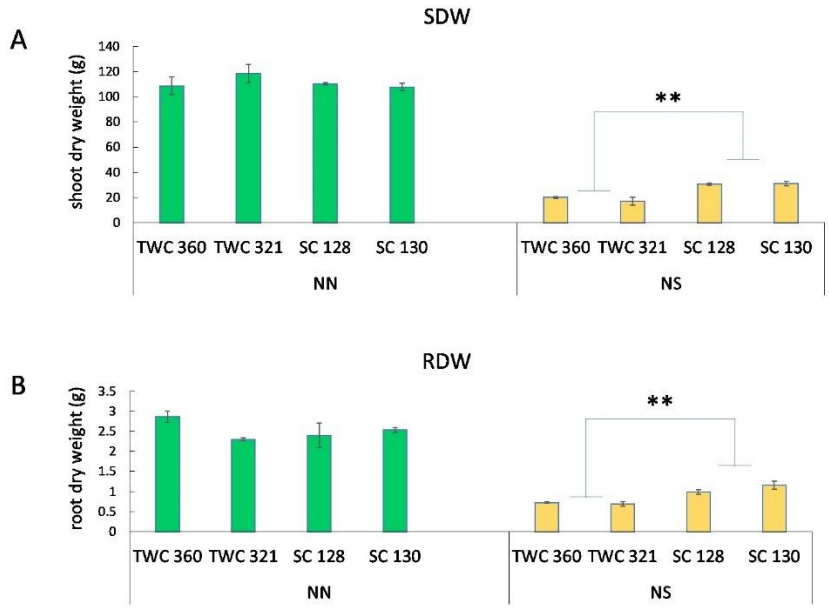

Fig. 2. The effect of N starvation stress of shoot dry weight or SDW (A) and root dry weight or RDW (B). Values represent the means of three replications ±SE. *Significant difference at P<0.05; **significant difference at P<0.01 in Duncan's multiple range test.

3.3 Root anatomical traits in N starvation stressed maize hybrids

Root anatomical traits, cross-sectional area (CSA), and stele area (SA) were examined in all selected maize hybrids under normal nitrogen (NN) and nitrogen starvation stress (NS). Figures 3 and 4 show that nitrogen starvation stress significantly reduced CSA and SA in all examined maize plants. The more sensitive maize hybrids, TWC 360 and TWC 321, had the lowest cross-sectional areas, 5.7 x 10^5 and 6.5 x 10^5 µm², respectively. In contrast, the less sensitive hybrids, SC 128 and SC 130, exhibited larger cross-sectional area values of 8.5 x 10^5 and 9.5 x 10^5 µm² (Fig. 3A). SA or stele area values showed a similar pattern (Fig. 3B). The effect of nitrogen starvation stress on the general architectural shape of an example of examined Egyptian maize hybrids is presented in (Fig. 5).

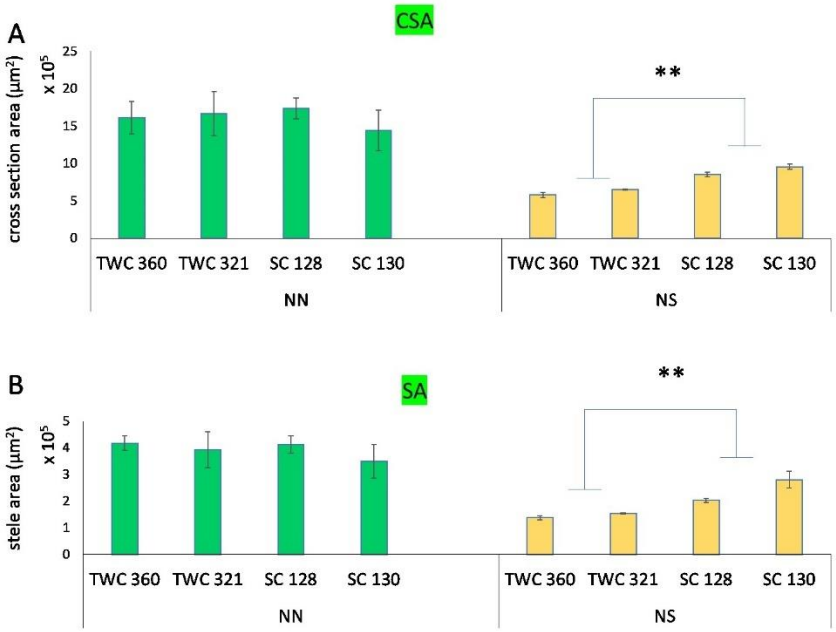

Fig. 3. The effect of N starvation stress of cross section area or CSA (A) and stele area or SA (B). Values represent the means of three replications ±SE. *Significant difference at *P*<0.05; **significant difference at *P*<0.01 in Duncan's multiple range test.

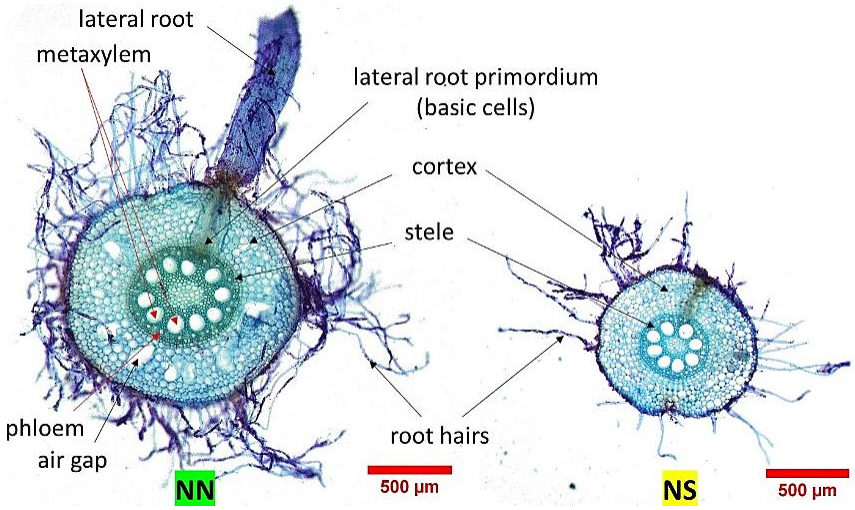

Fig. 4. Representaion to the effect of N starvation stress on the anatomical traits of seminal roots of maize hybrid TWC 321 at the age of one month. Toluidine Blue O staining (1.5%) of hand-made cross sections of fresh basal seminal root segments (5 cm from root base).

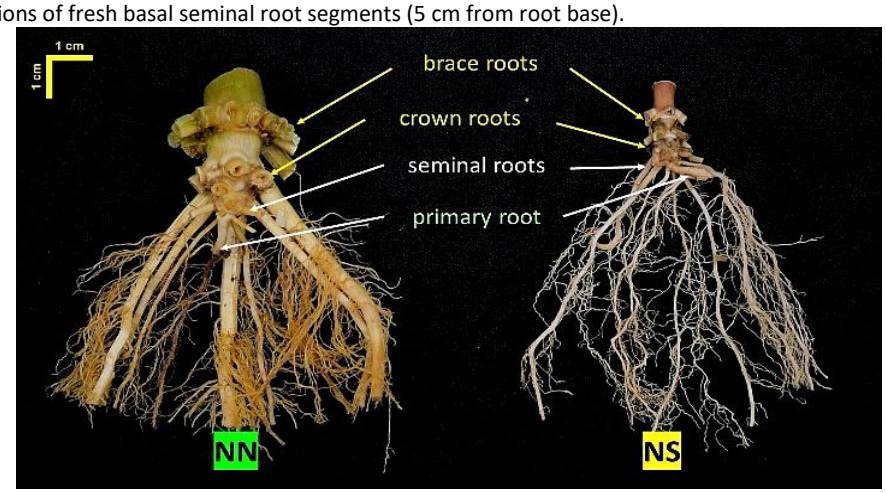

Fig. 5. Represention to the effect of N starvation stress of root architectural traits of Egyptian hybrid TWC 321 at the age of 3 months.

Total Chlorophyll content was elevated in less-sensitive hybrids under N stress:

Total chlorophyll content was spectrophotometrically estimated in leaves of all treated maize hybrids under control and N starvation stress (Figure 7). The applied N stress (starvation) led to a significant reduction in total chlorophyll content in the more sensitive maize hybrids TWC 360 and TWC 321 but not in the less sensitive hybrids SC 128 and SC 130. The total chlorophyll content in SC 128 and SC 130 (1.4 and 1.5 mg/g Fw) was significantly higher than in TWC 360 and TWC 321 (0.9 and 0.8 mg/g Fw).

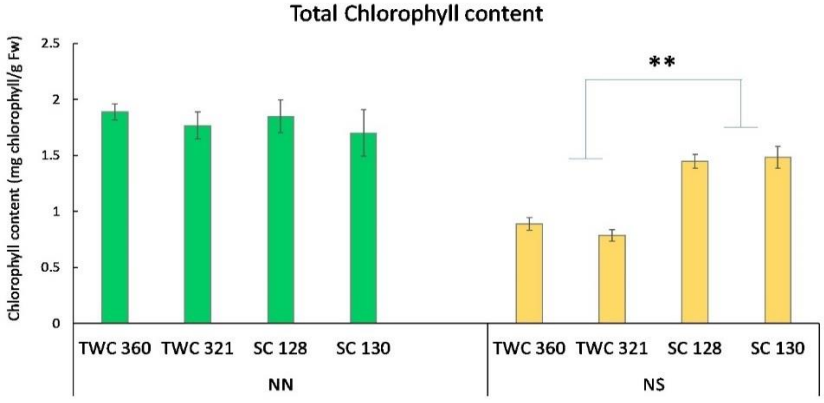

Fig. 6. The effect of N starvation stress on total chlorophyll content. Values represent the means of three replications ±SE. *Significant difference at *P*<0.05; **significant difference at *P*<0.01 in Duncan's multiple range test.

N starvation alters gene expression in maize hybrids leaves:

To assess the adaptation of maize plants to nitrogen starvation at the molecular level, the expression of several nitrogen deficiency stress marker genes was examined. In Fig. 7, the relative expression of catalase, amino acid permease 3, and high-affinity transferase 2.3 genes was quantified in response to nitrogen starvation stress.

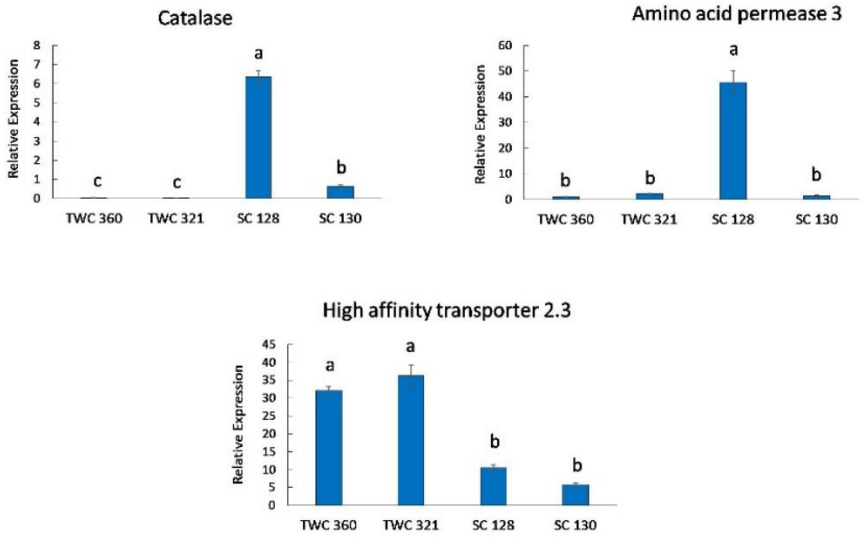

Fig. 7. The relative expression profile of selected stress marker genes in response to nitrogen stress in shoots of Egyptian maize hybrids. Values represent the means of three replications ± SE. Means with the same letters are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

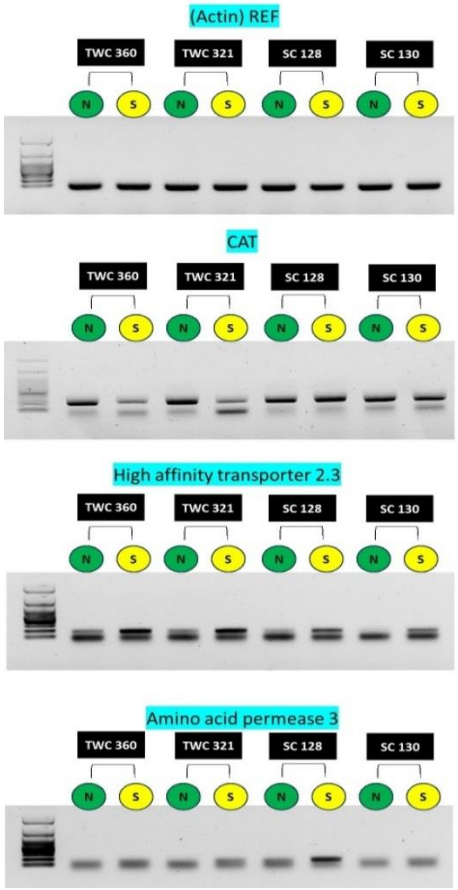

Fig. 8. Agarose gel electrophoresis (inverted color image) showing the migration of PCR product of each studied target gene and the reference gene. N for normal nitrogen growth conditions and S for nitrogen starvation stress.

It was found that the catalase gene was upregulated in both SC 128 and SC 130, with a mean ratio of a 130-fold increase compared to the more sensitive hybrids TWC 360 and TWC 321. The gene amino acid permease three was strongly upregulated in the less sensitive hybrid SC 128, with a mean value of a 42-fold increase relative to TWC 360, TWC 321, and SC 130. On the other hand, the less sensitive hybrids to nitrogen starvation stress, SC 128 and SC 130, significantly downregulated the expression of the high affinity transporter 2.3 gene with a mean reduction ratio of 76%.

Furthermore, we investigated the relative expression of genes: basic endochitinase, nodulin-related protein 1, and nitrate transferase (Fig. 10 and 11). In response to nitrogen starvation stress, the less-sensitive maize hybrids SC 128 and SC 130 accumulated more mRNA molecules of basic endochitinase and nitrate transferase 1 gene than the more-sensitive hybrids TWC 360 and TWC 321, with mean values of a 2.6-fold and 4-fold increase, respectively. The less-sensitive maize hybrid SC 130 accumulated more transcripts of the nodulin-related protein 1 gene than the two more-sensitive maize hybrids (TWC 360 and TWC 321) and the less-sensitive hybrid SC 128, with a mean value of a 4-fold increase.

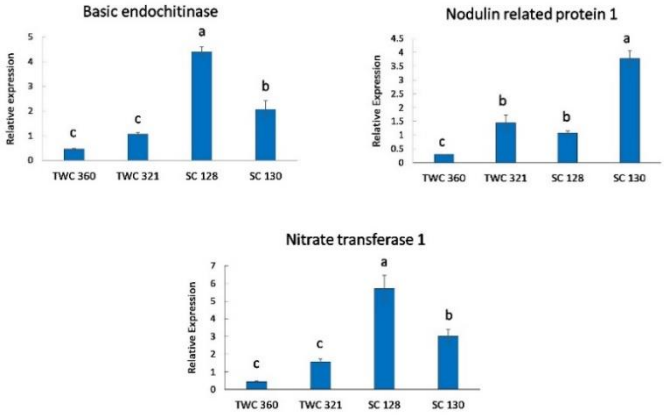

Fig. 9. The relative expression profile of selected stress marker genes in response to nitrogen stress in shoots of Egyptian maize hybrids. Values represent the means of three replications ± SE. Means with the same letters are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

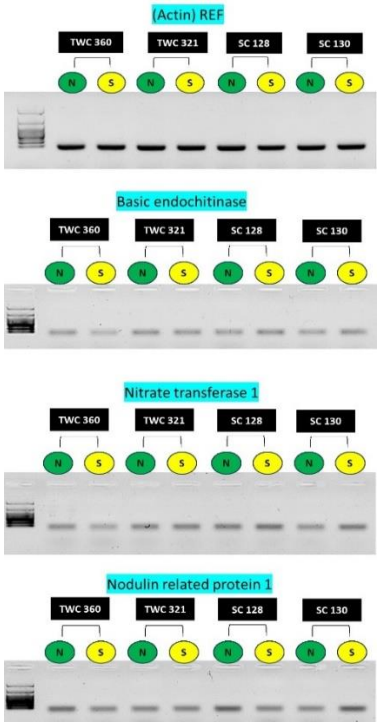

Fig. 10. Agarose gel electrophoresis (inverted color image) showing the migration of PCR product of each studied target gene and the reference gene. N for normal nitrogen growth conditions and S for nitrogen starvation stress.

DISCUSSION

Nitrogen (N) is the primary nutritional macro-element for photosynthetic capacity, thus influencing growth and productivity yield in maize (Urban *et al.,* 2021; Ostria-Gallardo *et al.,* 2024). Nitrogen fertilizers are extensively applied to maize fields for optimum yield because maize is a highly extractable crop for nitrogen (Sheoran et al., 2021). However, excessive applications of N fertilizers in agroecosystems are associated with nitrogen toxicity in water and air, leading to negative consequences on human health and climate change. Therefore, developing new maize genotypes with high nitrogen use efficiency (NUE) is considered an effective strategy for maintaining optimal maize crop production while using less nitrogen fertilizers, thereby saving on environmental impact and input costs (Xia and Yan, 2023). To achieve this challenging win-win strategy, understanding the molecular response of maize to nitrogen deficiency stress is essential. In this work, we screened several Egyptian maize hybrids with varying responses to nitrogen starvation stress to reveal how maize adapts to low-nitrogen agroecosystems.

Based on the external symptoms of severe nitrogen deficiency in the screened 12 Egyptian maize hybrids (data not shown), we selected two hybrids to represent the more sensitive ones (TWC 360 and TWC 321) and two hybrids (SC 128 and SC 130) to represent the less sensitive. As expected, nitrogen stress significantly reduced shoot dry weight and root dry weight in all examined maize plants. However, SC 128 and SC 130 hybrids appeared to use nitrogen more efficiently than TWC 360 and TWC 321 (Fig. 1 and 2). Wang *et al.* (2022) classified maize plants as efficient or inefficient in terms of nitrogen use efficiency (NUE) based on the reduction ratios of shoot dry weight (SDW). The most efficient plants had the lowest reduction ratios, while the most inefficient ones showed the largest reductions. We speculate that SC 128 and SC 130 possess high NUE, whereas TWC 360 and TWC 321 have lower NUE. This assumption is further supported by the estimated total chlorophyll content in leaves, where SC 130 showed the highest chlorophyll content under NS compared to TWC 360 and TWC 321 (Figure 6). It is well known that nitrogen deficiency leads to a severe reduction in chlorophyll content and, thus, photosynthetic capacity in maize (Mu and Chen, 2021).

To assess the molecular response of nitrogen stress tolerance in maize, we quantified the gene expression profile of several key stress marker genes related to nitrogen uptake, assimilation, and reallocation under nitrogen starvation stress (Fig. 7-10). The less-sensitive (more tolerant) Egyptian maize hybrids (SC 128 and SC 130) showed higher relative expression of the catalase gene, a basic antioxidant enzyme, indicating better antioxidative power under nitrogen starvation stress. The N stress triggers the overproduction of reactive oxygen species (ROS) like hydrogen peroxide and superoxide anions to toxic levels; the overexpression of several antioxidant enzyme genes, such as the catalase gene, is associated with a better adaptive response (Wang *et al.,* 2021). Basic endochitinase gene expression was also increased in the two less-sensitive hybrids compared to more-sensitive maize hybrids. Although not experimentally evaluated, it is speculated that SC 128 under N starvation growth conditions could be less susceptible to fungal pathogens (Abdul Haseeb *et al.,* 2022). We assume that NS-resilient maize hybrids (SC 128 and SC 130) might be better at detoxifying ROS and providing immunity against fungal pathogens than NS-sensitive maize genotypes (TWC 360 and TWC 321).

Plants have evolved two types of nitrogen (mainly $NO3^-$) uptake systems: the high-affinity transport system (HATS) for low external $NO3^-$ concentrations and the low-affinity transport system (LATS) for high external $NO3^-$ concentrations (Ohkubo *et al.,* 2021). Interestingly, under prolonged nitrogen starvation, both less-sensitive maize hybrids (SC 128 and SC 130) accumulated more transcripts of the Nitrate transferase 1 gene (high affinity to nitrogen), yet with lower expression of the high-affinity transporter 2.3 (Fig. 7 and 8) relative to TWC 360 and TWC 321. This observation suggests that less-sensitive maize plants might have a better ability to uptake N from the soil than more-sensitive plants through their roots due to the high relative expression of Nitrate transferase 1 (Singh *et al.,* 2022). It is speculated that the high-affinity transporter 2.3 expression could be related to the duration of exposure to N starvation stress. Further investigations could focus on quantifying gene expression under NS stress at different time points, such as early and adaptive responses.

Maize plants adopt various strategies for coping with nitrogen starvation in soil, which appear to be genotype-dependent. In this study, we noted that SC 128 accumulated more Amino Acid Permease 3 mRNA, indicating a better potential to transport amino acids from the site of synthesis to the site of activity (Zhou *et al.,* 2020). Conversely, SC 130 expressed the early responsive Nodulin-related protein 1 gene, reflecting this hybrid's suitability for breeding programs aimed at enhancing biological nitrogen fixation (BNF) in maize. BNF is a legume-based strategy where diazotrophs convert atmospheric nitrogen (N≡N) into a plant-available form, ammonium (NH4+). Some reports suggest that transferring this strategy to cereals like maize presents a promising opportunity to minimize the use of chemical nitrogen fertilization (Denancé *et al.,* 2014; Sheoran *et al.,* 2021).

Conclusion

Revealing the molecular response of how maize plants efficiently adapt to low nitrogen soil is key to achieving true N stress-tolerant maize lines with better nitrogen use efficiency (NUE). Our study showed that Egyptian maize genotypes with better resilience to N stress upregulated several stress marker genes associated with improved uptake, assimilation, and reallocation of nitrogen (N) from the soil. Revealing the kinetic response of N stress marker genes in maize at several time points is a future research focus. We emphasize that further studies are needed to apply such valuable molecular knowledge to maintain maize crop production at an optimum level for national food security while keeping the environment free from nitrogen pollution.

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Conflict of Interest: The authors declare no conflict of interest

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الكشف عن الاستجابة الجزيئية لتأقلم نبات الذرة تحت إجهاد نقص عنصر النتروجين في التربة الرملية منخفضة الخصوبة

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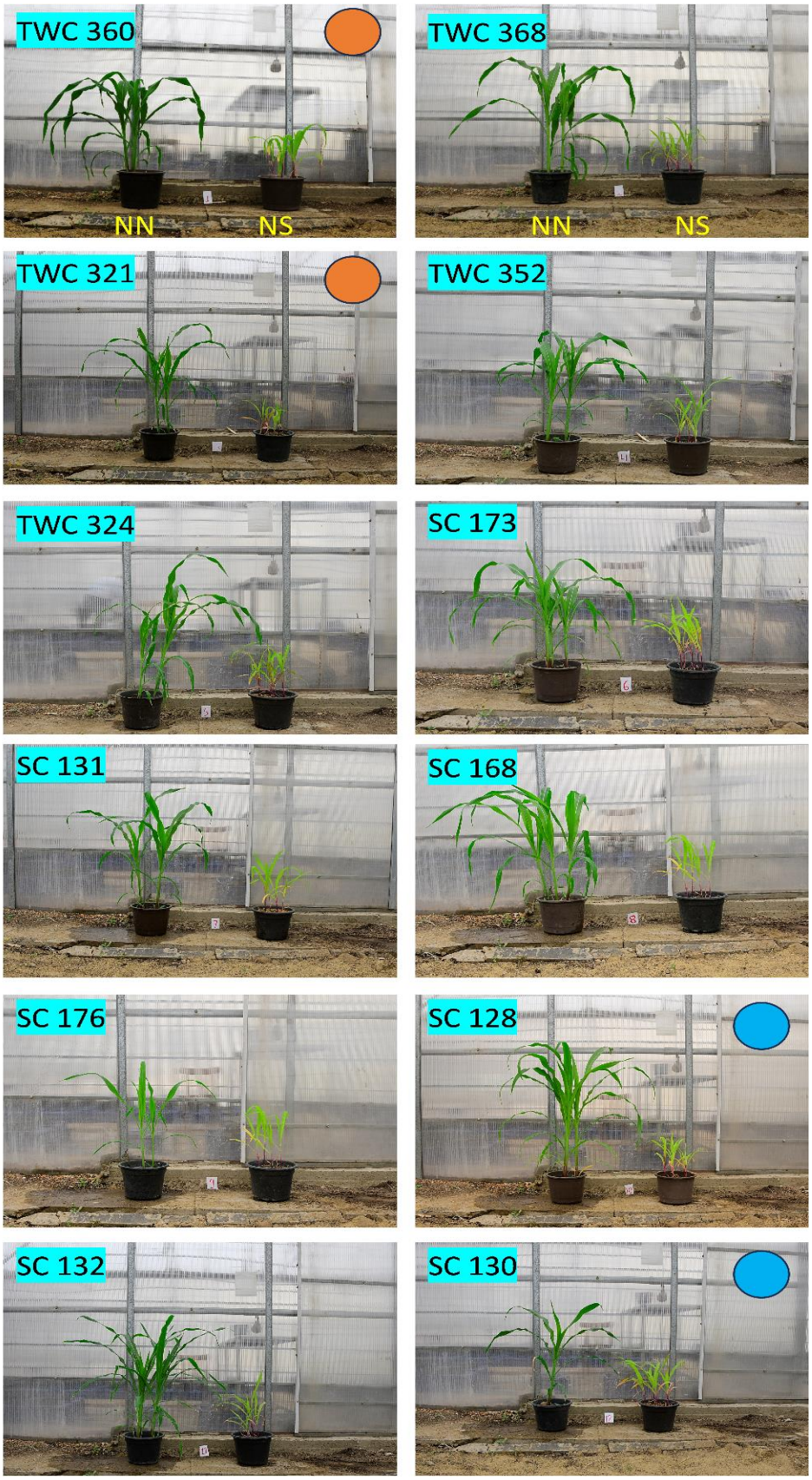
يعد تطوير هجن ذرة ذات مرونة بيئية تحت تأثير إجهاد نقص عنصر النتروجين بالتربة إستراتيجية مستدامة من اجل خفض تكلفة الأسمدة النيتروجينية وكذلك تلوث كل من التربة والهواء بالبيئة الزراعية المصرية. في هذا العمل البحثي تم التقييم الظاهري لعدد اثنا عشر هجين ذرة مصري لإجهاد نقص عنصر النتروجين بالتربة وبناء على ذلك تم اختيار اربعة هجن ذات استجابات متباينة وهم الهجن الفردية 128 و130 لتمثل الأقل تأثرا (الأكثر تحملا) والهجن الثلاثية 321 و360 لتمثل الأشد ضررا نتيجة الغياب الكامل لعنصر النتروجين بالتربة. في حالة الحرمان التام للتسميد النتروجيني لمدة ثلاثة أشهر لوحظ ان الوزن الجاف لكل من السيقان والجذور تأثرا سلبا في كل الهجن المختبرة، وقد كان التأثير الأقل بشكل معنوي في حالة الهجين الفردي 130 ليكون 71% و54%، على التوالي. أما في حالة الجذور، فأن مساحة القطاع العرضي للجذر تأثرت سلباً بنسبة 64% و61% في حالة كل من الهجين الثلاثي 360 و321، ولكن تلك النسب كانت 50% و31% فقط في حالة كل من الهجين الأحادي 128 و130، على التوالي. وفي حالة كمية الكلوروفيل الكلي وجد انها في حالة الهجين الفردي 130 (الأقل تضررا) كانت تقريبا 2 ميليجرام لكل جرام وزن طازج، أما في حالة الهجين الثلاثي (الأكثر تضررا) فقد كانت 0,97 ميللجرام لكل جرام وزن طازج تحت تأثير إجهاد النتروجين بالتربة. تم قياس التعبير الجيني الكمي لعدد من الجينات المرتبطة بتأقلم نبات الذرة تحت تأثير اجهاد النقص التام للتسميد النتروجيني بالتربة الرملية منخفضة الخصوبة. بالنسبة للجينات Catalase, Nitrate transferase 1 و Endochitenase كان التعبير الجيني أكبر بشكل معنوي في أوراق كل من الهجين الفردي 128 و130، ولكن كان التعبير الكمي للجين High affinity transporter 2.3 أقل فيهما مقارنة بالهجن الثلاثية 321 و360 (الأشد تضررا).

الكلمات المفتاحية: الذرة.؛ إجهاد نقص عنصر النتروجين ؛ التعبير الجيني؛ سمات الجذر

Appendix (Supp. 1).



Supp. 1. Phenotypical response of all examined 12 Egyptian maize hybrids in response to N starvation stress and selecting two more-sensitive hybrids (TWC 360 and TWC 321, orange circles) and two less-sensitive hybrids (SC 128 and SC 130, blue circles).