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Metabolic Responses to Contrasting Levels of N Fertilization during Ear Leaf Transition from Assimilation to Remobilization at Grain Filling in Maize

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MAIZE grain yield is shaped by source-sink relationships during grain filling. Such relations are driven by complex metabolic changes, responsive to N availability and are not fully understood. Here, the impact of limited and sufficient N fertilization on the metabolic interconversions in the ear leaf of a new maize field-grown hybrid (Tzi8 × Mo17) during its critical transition from assimilation to remobilization were chronologically investigated at 0, 5, 10, 15, and 20 days after pollination. N-deficient plants produced 43% less grain yield and 13% less biomass however, they had 51% higher root dry weight and higher Root/Shoot ratio at anthesis. The low N-induced reduction in yield and biomass accumulation was associated with earlier chlorophyll degradation and overall decrease in leaf chlorophyll, total soluble proteins, carbon (C: sucrose & total soluble sugars), and N assimilates (leaf N, nitrate, ammonia & amino acids). In contrast, N-deficient plants accumulated 18% more starch and 24 % flavonoids than N-sufficient plants and such responses were driven by low N-induced sink limitation. N-deficient plants also had significantly higher activities of N remobilizing (asparaginase & protease) but lower activities of N assimilating (nitrate reductase & glutamine synthetase) enzymes compared to N-sufficient plants. Glutamate and aspartate followed by branched amino acids dominated the amino acids pool under both N conditions. Altogether, the accumulation of starch and flavonoids and the induction of N remobilizing enzymes represent low N-specific responses whereas the rest of responses depict the common metabolic interconversions between adequate and limited N-induced responses during maize grain filling.

Keywords: Enzymes, Flavonoids, Maize, Metabolites, Nitrogen, Yield.

Introduction

Maize (*Zea mays* L.) is a leading cereal crop and a primary staple food for the expanding population (Zhang et al., 2016). Grain yield of maize is largely dependent on the ear leaf growth within the 2-3 week period around silking (Seebauer et al., 2004; Ning et al., 2018a). During this stage, coordination between production of photoassimilates in source leaves and the developing sink is established to secure recourses for grain filling (Tollenaar et al., 1992; Lawlor & Paul, 2014; Ning et al., 2021). Such cross-talk drives differential biomass allocation to various plant organs including grains (Marcelis, 1996; Lee & Tollenaar, 2007). About 50% of maize dry matter is allocated to grains and therefore dry matter accumulation and allocation is one of the major determinants of grain yield in maize hybrids (Lee & Tollenaar, 2007; Ning et al., 2013; Tao et al., 2014).

Source capacity is determined by the photosynthetic activity of leaves and the availability of carbohydrates for transport (Uhart & Andrade, 1995; Westgate et al., 2004). On the other hand, sink capacity is defined mainly by the number and

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size of developing kernels on the ear (Borrás & Westgate, 2006; Bu et al., 2013). High sink/source ratio in plants reflects more photosynthetic activity and enhanced remobilization from source to sink organs (Rogers et al., 1995). Both source capacity and sink strength are significantly influenced by nitrogen (N) availability. N limitation reduces sink capacity via induction of abortion of pollinated ovules (Lemcoff & Loomis, 1994). It also decreases the photosynthetic activity of source leaves and consequently carbohydrate production (Masclaux-Daubresse et al., 2010; Albacete et al., 2014). Therefore, N limitation eventually disturbs source-sink relationships and consequently results in significant reduction in grain yield (Martin et al., 2006; Ning et al., 2018b).

In maize, leaf N is positively correlated to grain yield (Kovács & Vyn, 2017). Leaf N can be present in various forms such as proteins, ammonia, nitrate, and amino acids. Total leaf N and total soluble proteins (TSP) are used as indicators to the overall plant N status with respect to N assimilation and remobilization (Jeuffroy et al., 2002; Hirel et al., 2005b). Free amino acids and ammonia content are biomarkers for the metabolic activity of plants during the transition of N metabolism from assimilation to remobilization (Masclaux et al., 2000; Masclaux-Daubresse et al., 2017). The leaf-derived amino acids represent about 50-80% of N needs of reproductive organs in maize (Masclaux et al., 2001; Christophe et al., 2011). Degradation of leaf chlorophyll is a main biomarker for resource remobilization and progress of leaf senescence during grain filling. The efficient synthesis, recycling, and remobilization of the above physiological markers and metabolites during reproductive stage is the driving force for efficient grain filling (Gallais et al., 2006; Masclaux-Daubresse et al., 2008). Therefore, monitoring the alterations in these biomarkers during grain filling under field conditions has been an interesting approach for clues on source/sink cross-talk and on the physiological interconversions undelaying transition of leaf metabolism from assimilation to remobilization under environmental conditions of interest (Hirel et al., 2005a; Martin et al., 2006; Yu et al., 2016).

Kernel set and grain filling are tightly linked to dynamics of enzymes central to N metabolism (Martin et al., 2006; Yu et al., 2016). Nitrate reductase (NR) and glutamine synthetase (GS) are key enzymes in N metabolism and both drive critical steps in N assimilation in maize leaves. NR catalyzes the reduction of nitrate to nitrite as the first committed step in N assimilation pathway and such assimilatory activity becomes very limited after silking under field conditions (Kaur et al., 2019). Leaf GS drives the assimilation of ammonia released from various sources in cytoplasm (GS_{1-4}) and plastid (GS_2) and is a crucial checkpoint for controlling growth and productivity (Simons et al., 2014; Thomsen et al., 2014). The activities of NR and GS are responsive to N availability, organs, and developmental stages (Hirel et al., 2005a,b). Other critical enzymes such as protease and asparaginase (ASNase) drive the hydrolysis of leaf proteins and asparagine, respectively as the grain filling progresses. Their induction in leaf is a critical physiological marker on the onset of resource remobilization from leaves to grain and their activity in chloroplast is tightly regulated by N remobilization during leaf senescence (Xu et al., 2012).

In the current study, we cross-pollinated the two maize inbreds Tzi8 and Mo17 to generate a new Tzi8 x Mo17 hybrid. These inbreds have unique N use-related characteristics. Tzi8 exhibits an excellent grain yield under high N but is sensitive to low N highlighting its potential use in dissection of physiological responses to high and low N availability (Edmeades et al., 1996). In addition, Tzi8 was a parent of one of the most rapid grain filling hybrids in N deficient soil under Egyptian agricultural conditions (Ibraheem & Abdel-Moneam, 2015). Similarly, Mo17 is responsive to high and low N fertilization rates (Ibraheem & El-Ghareeb, 2020). Also, Mo17 was a parent of many high yielding maize hybrids and was used to develop one of the principal biological materials for gene discovery (Intermate B73xMo17 Recombinant Inbred Lines; IBMRIL) in maize (Lee et al., 2002). Therefore, the hybrid Tzi8 x Mo17 provides a unique plant material to monitor pollination-induced metabolic interconversions that drive source/sink relationships during maize grain filling under sufficient and deficient N fertilization rates. Up to our knowledge, this may be the first report on the physiological responses of the ear leaf of the high yielding Tzi8 x Mo17 to contrasting levels of N availability during leaf transition from assimilation to remobilization at grain filling. Our hypothesis is that contrasting N fertilization rates, during this critical period, would have pronounced effects on the availability and the mutual interaction of C and N metabolites in ear leaf

and their transport to kernels, biomass allocation to grains, nitrogen use efficiency (NUE) and yield. In addition, ear leaves (the leaf subtending the cob) of N-deficient and N-sufficient Tzi8 x Mo17 plants may be programmed to maintain common and specific patterns of dynamics of metabolic changes in ear leaf during its transition from assimilation to remobilization. Therefore, the objectives of the current study were to evaluate the impacts of contrasting N fertilization rates on the metabolic status of ear leaf as grain filling proceeds and link that with the concomitant changes in source-sink relationships. This was carried out by monitoring chronological changes in the dynamics of critical carbon (C) and N metabolites, enzymes involved in N assimilation/remobilization and N stress-related secondary metabolites in ear leaf at 0, 5, 10, 15, and 20 Day After Pollination (DAP) under limited and sufficient N fertilization. Grain yield, NUE and their related traits in N-sufficient and N-deficient plants were also monitored to extend the physio-agronomic significance of the study. Finally, correlation among various metabolites was performed to test their linkage regardless N availability.

Materials and Methods

Genetic stocks

Seeds of two maize inbreds, Tzi8 and Mo17, were obtained from Dr. Stephen Moose, at the University of Illinois, USA. Our initial evaluation of these inbreds revealed synchronized flowering and maturation under the Egyptian agricultural conditions which facilitates the production of the new Tzi8 × Mo17 hybrid by the authors. Enough grains of the hybrid were produced and used in the current study.

Experimental design and treatments

Field experiments were carried out in a N responsive nursery during summer of 2017 and 2018. The soil of the experimental site was clayey in texture with 2.68 % organic matter, 18ppm available N, 7ppm available P, 245ppm available K, and pH of 7.9. The experimental design was split plot design. The experimental field was divided into four main plots: two for low N and two for high N rates. Each plot had 10 rows of 3 m long and spaced 70cm apart. Seeds were hand-sown, two seeds per hill, on the 13th of May 2017 and 10th of May 2018. Within a row, plants were 0.25m a part and thinned to one plant per hill when the second leaf had a visible collar (a stand density of 57142 plants/ha). Plants were grown under two rates of N fertilizer (low N: 71.4kg N/ha and high N: 285.7kg N/ha). The fertilizer was applied once down the center of the row as ammonium nitrate. Plots were kept weed-free manually and were irrigated every 10-12 days till maturity.

Data collection

Biomass accumulation, allocation and yield

Growth, biomass accumulation/allocation, and physiological responses were performed on plant samples collected every 5-day intervals through the first 20 DAP whereas yield and yield components were carried out on mature plants. For biomass accumulation, three plants per plot were harvested and separated into ear leaves, other leaves, stalks (with leaf sheath and tassels), and ear (husk, cobs, and grains). The samples were dried in an oven at 65^{0}C to a constant weight. Dry weights were recorded and used to assess dry matter allocation to different plant organs and aboveground biomass production. Post-silking biomass accumulation was calculated as the difference in dry matter accumulation between samples harvested at 0 and 20 DAP. Based on dry weight of ear, its biomass partitioning coefficient (PC) was calculated using the equation [PC= 100× (Ear dry weight / Total dry weight of plant)] (Krishnan et al., 1998). At silking, three plants were uprooted to determine root and shoot dry weights as well as root to shoot (R/S) ratio.

At physiological maturity (indicated by a black spot on kernel/cob attachment) biological yield was estimated. Mature ears were then harvested, dried, and used to estimate grain yield (gm/ plant), 100-kernels weight (gm), and kernels number per ear. The plant source-sink ratio (SSR) was calculated as ratio of post-silking biomass per plant to kernel number per plant (Chen et al., 2016). NUE components were calculated according to (Moll et al., 1982): Nitrogen uptake efficiency (NUpE) was calculated as the ratio of total N in the aboveground biomass (kg/ha) to supplemented N (kg/ha) and nitrogen utilization efficiency (NUtE) was estimated as the ratio of grain yield (kg/ha) to total N (kg/ha) in the aboveground biomass. NUE was calculated as the ratio of grain yield (kg/ha) to supplemented N (kg/ha).

Metabolites analysis

Chlorophyll

Chlorophyll in ear leaves was extracted in 80% cold acetone and the concentration was determined

spectrophotometrically at 663, 647, and 470nm (Lichtenthaler & Wellburn, 1983).

Carbohydrates

Ear leaves were collected and dried at 65^{0}C to a constant weight. The midribs were removed and the leaves were ground into fine powder and used for extraction and determination of different metabolites. 100mg of the powdered ear leaves was extracted in 80% ethanol, centrifuged, and the clear extracts were used for determination of sucrose and total soluble sugars (TSS) using anthrone method (Yemm & Willis, 1954; van Handel, 1968). Sugar free residues were dried over a water bath, hydrolyzed using 52% perchloric acid, and the released sugars were determined using anthrone method spectrophotometrically at 630nm (Hedge & Hofreiter, 1962).

N fractions and amino acids

Total N concentration was measured using NC analyzer (Model flash 2000, serial number 2015, F0028). N fractions in the powdered dried tissues were extracted in water as described by Yemm & Willis (1956) and the aqueous extracts were used for spectrophotometric determination of nitrate (Cataldo et al., 1975) and ammonium (Delory, 1949). Amino acids profiles in pooled samples of ear leaves of three biological replicates were measured at 0 and 10 DAP under both low and high N levels according to (AOAC, 2012) using amino acid analyzer (Biochrom 30; Biochrom Ltd., Cambridge Science Park, Cambridge, England).

Protein and enzyme assays

Three additional ear leaves from each N treatment were detached from plants, cleaned, frozen immediately in liquid N and stored at -80^{0}C. Total soluble proteins (TSP) were extracted in a buffer containing 50 mM Tris-HCl, 1mM EDTA, 1mM DTT, and pH 7.8 and measured by dye binding assay using Coomassie Brilliant Blue (G-250) (Bradford, 1976). The activities of NR, GS, ASNase, and protease enzymes were determined in the above protein extract. NR activity was estimated according to Reed et al. (1980) and expressed as unit/ mg protein. One unit corresponds to the production of 1µg of nitrite/minute. GS activity was measured according to O'neal & Joy (1973) and was expressed as unit/ mg protein. One unit corresponds to the production of 1µg of glutamyl hydroxamate/min/mg protein. ASNase enzyme catalyzes the conversion of asparagine to aspartic acid and ammonia. Its activity was assayed according to Ren et al. (2010) and expressed as unit/ mg of protein. One unit of ASNase activity is defined as the amount of the enzyme that liberates 1µmol of ammonia/min/mg protein. Protease activity was determined following procedures of Lai & Liao (1974) using 1% casein as a substrate and ninhydrin reagent. Its activity was expressed as unit per mg of protein. One unit was expressed as mg of azocasein degraded by mg protein/min.

Anthocyanins and flavonoids

Anthocyanins in ear leaves were extracted by suspension of 0.1gm of powdered dry leaves in 10mL of acidified methanol (methanol: water: HCl; 79:20:1, V/V.) at 0ºC for 72hrs in the dark with continuous shaking. After centrifugation, the absorbance was measured at 530 and 657nm (Mancinelli et al., 1975). Anthocyanin levels were expressed as mg cyanidin-3-glucoside/gm dry weight using the reported molar extinction coefficient of 25900M^{-1} cm^{-1} and a molecular weight of 449.2gm/mol. Flavonoids in ear leaf were extracted in 50 % methanol and total flavonoids content were then determined by $AlCl_3$ method using spectrophotometer at 510nm and Quercetin as a standard (Atanassova et al., 2011). Results were expressed as mg quercetin/gm dry weight.

Statistical analysis

The data of the tested parameters are presented as means of three biological replicates at each time point. Data of N treatment-induced responses were evaluated by one-way ANOVA using COHORT/ COSTAT software (798 Lighthouse Ave. PMB 329, Monterey, CA, 93940, USA). Data were normalized using Shapiro-Wilk test. Means were compared using the least significant difference test (LSD) at P value (5%) to test the statistical significance. Yield data were averaged across the two years. Pearson correlation was conducted using statistical software "Past" version 4.02.

Results

Performance of Tzi8 x Mo17 plants under contrasting N fertilization rates

To test the impact of limited and sufficient N fertilization on the performance of Tzi8 x Mo17 plants, biological yield, grain yield, its components, SSR ratio and NUE were monitored at maturity. Biomass accumulation and allocation during the first 20 DAP were also tested. N limitation significantly reduced biological yield, grain yield, kernels number/ear, and 100- kernels weight (Table

1). N-deficient plants had 43% less in overall grain yield and 28.6% less in SSR than N-sufficient plants. Kernels number per ear was the most affected yield component by N limitation with ~ 26% reduction compared to sufficient N treatment. N-deficient plants had significantly less dry matter in leaves, stem, ear, aboveground, and post-silking biomass accumulation than N-sufficient plants (Table 2). Both N-deficient and N-sufficient plants increased their PC to ear as grain filling proceeded with consistently lower PC records in N-deficient than N-sufficient plants (Fig. 1). At silking, N-deficient plants had approximately two folds higher root dry weight and R/S ratio than N-sufficient plants. This was associated with about two folds higher NUE and NUpE along with 24.5 % increase in NUtE in N-deficient than N-sufficient plants (Table 3).

Changes in carbon metabolism-related biomarkers during grain filling

N limitation reduced leaf chlorophyll at all-time points after pollination. Slightly different patterns of chlorophyll dynamics throughout the first 20 DAP were noted under limited and sufficient N input (Fig. 2A). N-deficient plants had their maximum chlorophyll at pollination and gradually decreased thereafter whereas N-sufficient plants had their maximum leaf chlorophyll at 5 DAP and dramatically declined as grain filling progressed.

N-deficient plants had lower foliar sucrose than N-sufficient plants; however, both had similar dynamics in leaf sucrose as grain filling progressed (Fig. 2B). Ear leaves had their maximum sucrose from 5 to 10 DAP and significantly declined thereafter with more intense decline in N-sufficient than N-deficient plants; particularly at 15 DAP. Ear leaf transported 21% less sucrose in N-deficient plants than N-sufficient plants. Similar to sucrose, N-deficient plants had lower leaf TSS than N-sufficient plants, except at 20 DAP where both had relatively similar TSS (Fig. 2C). The dynamics of leaf TSS relatively differed under both N rates. N-deficient plants had their maximum leaf TSS at 5 DAP and then gradually declined whereas N-sufficient plants had relatively constant level of TSS up to 15 DAP and declined thereafter. In contrast to sucrose and TSS, significantly higher leaf starch was observed in N-deficient plants compared to N-sufficient plants and such differences were more apparent at later stages (Fig. 2D). Under N limitation, leaf starch continuously increased as grain filling progressed, however, under high N, starch content increased up to 15 DAP then sharply decreased.

Anthocyanins and total flavonoids in ear leaf during grain filling

N-deficient Tzi8 x Mo17 plants had higher anthocyanins and total flavonoids than N-sufficient plants throughout the experimental period (Fig. 3A & B). Time course analysis revealed progressive increase in these secondary metabolites as leaf aged with significantly higher level in N-deficient plants than N-sufficient plants at most time points particularly at later stages.

TABLE 1. Biological yield, grain yield, kernels number/ear, 100-kernels weight, and source-sink ratio (SSR) in maize at maturity under contrasting levels of N fertilization

Treatment	Biological yield (gm/ plant)	Grain yield (gm/ plant)	Kernels number/ ear	100-kernels weight (gm)	SSR (mg/kernel)
Low N	264.65b±7.00	89.89b±**7.9**	304b±**10.23**	32.62b±1.3	269.37b±**18.50**
High N	358.27a±7.23	159.72a±7.1	411a±**9.12**	42.49a±1.5	377.34a±**26.25**

- Numbers followed by different letters indicate significant differences between N treatments (P<0.05).
- Shown are means ±SD.

TABLE 2. Dry matter accumulation in the different plant parts and post-silking dry matter accumulation through 20 DAP in maize under contrasting levels of N fertilization

N treatment	Leaves dry matter (gm)	Stem dry matter (gm)	Ear dry matter (gm)	Above ground biomass (gm)	Post-silking dry matter accumulation (gm)
Low N	58.42b±0.37	115.47b±1.3	20.42b±0.3	194.59b±1.8	8.18b±1.3
High N	62.30a±2.6	128.61a±2.4	30.87a±2.05	223.18a±3.00	14.32a±2.7

- Numbers followed by different letters indicate significant differences between N treatments (P<0.05).
- Shown are means ±SD.

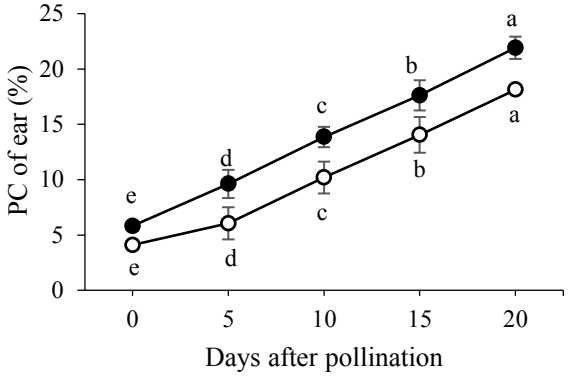

Fig. 1. Partition coefficient (PC) of ear from 0 DAP to 20 DAP at low N and high N treatments [Shown are the means of three biological replicates and error bars represent ± SD. Different letters indicate significant differences between samples within each N treatment at P<0.05. Closed symbols correspond to high N and opened symbols correspond to low N]

TABLE 3. Root dry matter accumulation, root/shoot ratio (R/S) at silking, and nitrogen use efficiency (NUE), nitrogen uptake efficiency (NUpE), and nitrogen utilization efficiency (NutE) at maturity

N treatment	Root dry matter (gm)	R/S	NUE (kg/ha)	NUpE (kg/ha)	NUtE (kg/ha)
Low N	23.13a±1.4	0.12a±0.006	71.93a±6.4	2.28a±1.7	31.75a±5.05
High N	15.30b±0.67	0.06b±**0.003**	31.94b±1.4	1.27b±1.7	25.49a±4.74

- Numbers followed by different letters indicate significant differences between N treatments (P<0.05).
- Shown are means ±SD.

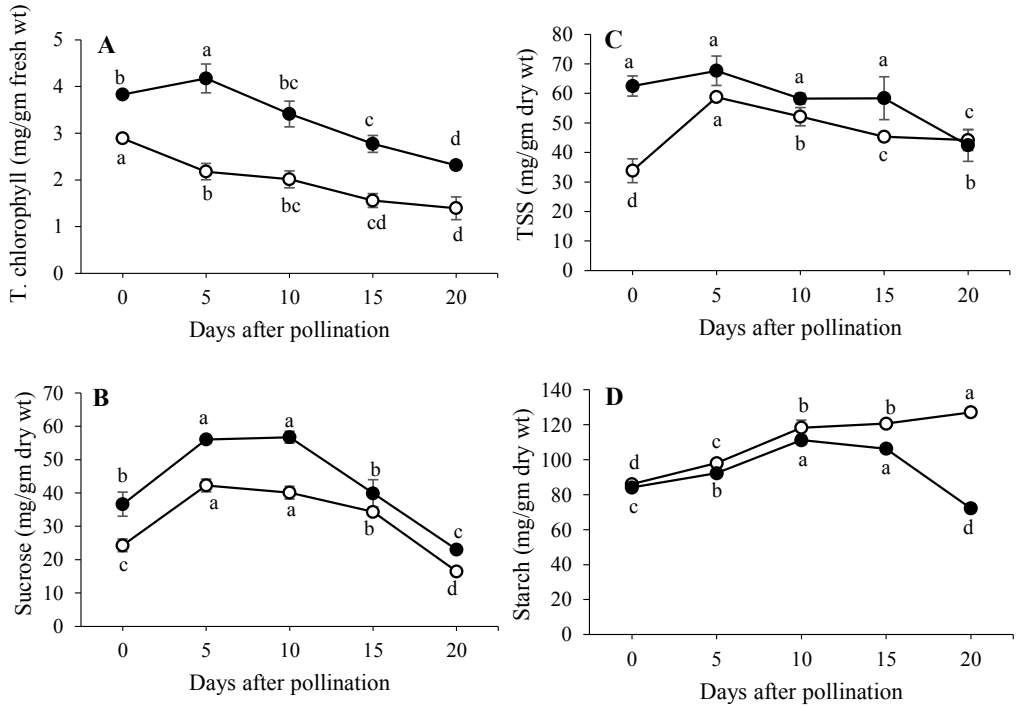

Fig. 2. Carbon metabolites: Total chlorophyll (mg/gm fresh wt) (A), sucrose (mg/gm dry wt) (B), total soluble sugars (TSS) (mg/gm dry wt) (C), and starch (mg/gm dry wt) (D) of ear leaf from 0 DAP to 20 DAP under low N and high N treatments [Shown are the means of three biological replicates and error bars represent ± SD. Different letters indicate significant differences among samples within each N treatment at P<0.05. Closed symbols correspond to high N and opened symbols correspond to low N]

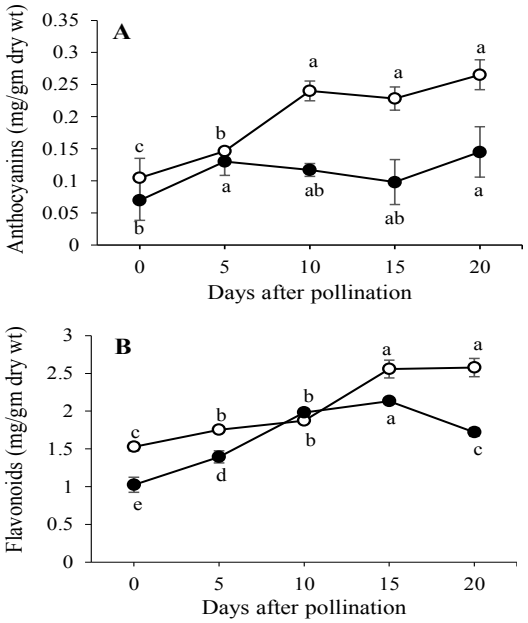

Fig. 3. Secondary metabolites: Anthocyanins (mg/gm dry wt) (A) and total flavonoids (mg/gm dry wt) (B) of ear leaf from 0 DAP to 20 DAP under low N and high N treatments [Shown are the means of three biological replicates and error bars represent ± SD. Different letters indicate significant differences among samples within each N treatment at P<0.05. Closed symbols correspond to high N and opened symbols correspond to low N]

Alterations in N metabolism-related biomarkers during grain filling

N limitation induced overall reductions in leaf N, nitrate, ammonia, and TSP at all-time points after pollination compared to adequate N fertilization (Fig. 4). Leaf N and nitrate had relatively similar dynamic patterns with maximum levels at pollination and a rapid decline as grain filling progressed, however the rate of decline in leaf nitrate was higher than leaf N (Fig. 4A & B). Also, N-deficient plants had their highest foliar ammonia at pollination and decreased afterward whereas N-sufficient plants tended to have maximum leaf ammonia at 5 DAP and decreased thereafter with significantly higher reduction at 20 DAP (Fig. 4C). Similar patterns of leaf TSP dynamics were observed under contrasting N treatments with maximum TSP at 5 DAP and gradual decline as leaf aged (Fig. 4D). Amino acids profiling at 0 and 10 DAP as two representative stages of ear leaf development revealed significant differences in total as well as composition of the amino acid pools in the ear leaves of N-deficient and N-sufficient plants. N-deficient plants had less total as well as individual free amino acids than N-sufficient plants (Table 4). Glutamate, aspartate, leucine, alanine, and valine dominated the amino acid pool in ear leaf regardless N treatment.

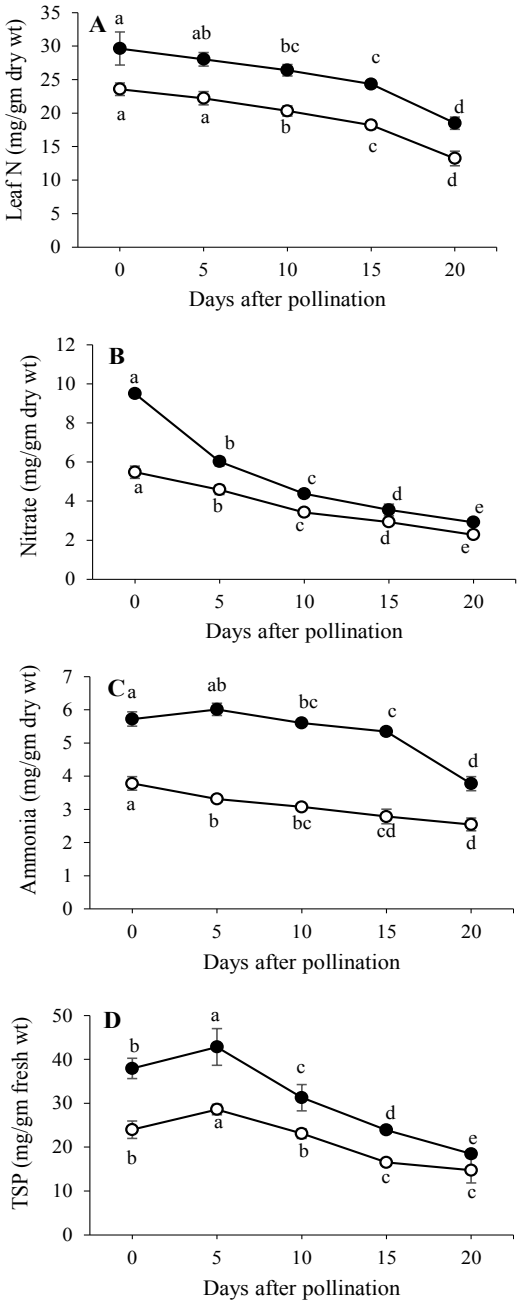

Fig. 4. N metabolites and protein: Leaf N (mg/gm dry wt) (A), nitrate (mg/gm dry wt) (B), ammonium (mg/gm dry wt) (C), and total soluble protein (TSP) (mg/gm fresh wt) (D) of ear leaf from 0 DAP to 20 DAP under low N and high N treatments [Shown are the means of three biological replicates and error bars represent ± SD. Different letters indicate significant differences among samples within each N treatment at P<0.05. Closed symbols correspond to high N and opened symbols correspond to low N]

TABLE 4. Amino acid profiles in ear leaf of N-deficient and N-sufficient maize plants at 0 and 10 days after pollination

Treatment / Amino acids	Low N		High N	
	0 DAP	10 DAP	0 DAP	10 DAP
Aspartate	1.17	1.04	1.34	1.36
Therionine	0.59	0.5	0.67	0.65
Serine	0.6	0.47	0.65	0.62
Glutamate	1.52	1.32	1.73	1.69
Glycine	0.64	0.56	0.72	0.72
Alanine	0.94	0.8	1.16	1.08
Valine	0.72	0.64	0.83	0.82
Isoluecine	0.59	0.53	0.66	0.67
Leucine	1.03	0.92	1.2	1.2
Tyrosine	0.45	0.4	0.56	0.53
Phenylalanine	0.66	0.6	0.81	0.79
Histidine	0.21	0.18	0.26	0.26
Lysine	0.49	0.41	0.61	0.54
Argnine	0.61	0.55	0.77	0.72
Proline	0.65	0.6	0.81	0.79
Cystine	0.13	0.2	0.21	0.24
Methionine	0.28	0.25	0.33	0.33
Total	11.28	9.97	13.32	13.01

Changes in activities of N assimilation and remobilization- related enzymes in ear leaf during grain filling under contrasting N fertilization.

N limitation suppressed the activities of NR and GS in ear leaves at all-time-points throughout grain filling (Fig. 5A & B). However, the low N-induced suppression of GS was remarkably higher than that of NR. The two enzymes showed different dynamics through the experimental period. Under both N rates, the highest NR activity was noted at pollination, sharply declined at 5 DAP, then either remained constant (high N) or sharply decreased (low N) as grain filling progressed. At later stages, N-deficient plants had about ~2-3 folds less NR activity than N-sufficient plants. Unlike NR and GS, N limitation increased the activity of leaf ASNase and protease (Fig. 5C & D). As grain filling proceeded, both N-deficient and N-sufficient plants increased the activities of both enzymes with relatively similar patterns up to 15 DAP after which the rate of increase either slowed down (protease) or declined (ASNase). At 20 DAP protease activity approached 2 and 4 folds higher than that at pollination under low N and high N, respectively.

Correlation among C and N metabolites, enzymes and secondary metabolites in ear leaf across N regimes

Regardless the level of N treatments and days after pollination, our correlation analysis revealed strong positive as well as negative correlations among the tested C and N biomarkers, N-assimilating and N-remobilizing enzymes and secondary metabolites in ear leaf during grain filling. For example, leaf N exhibited strong positive correlation to leaf TSP ($r^2 = 0.86^{***}$), chlorophyll ($r^2 = 0.91^{***}$) and activities of both NR ($r^2 = 0.81^{***}$) and GS ($r^2 = 0.84^{***}$) (Table 5). Strong positive correlations were also observed between NR activity and leaf nitrate ($r^2 = 0.89^{***}$) as well as between GS activity and leaf ammonia ($r^2 = 0.86^{***}$). On the other hand, starch, protease, ASNase, anthocyanins and flavonoids were positively correlated to each other; however, they exhibited negative correlations with almost all other metabolites (Table 5).

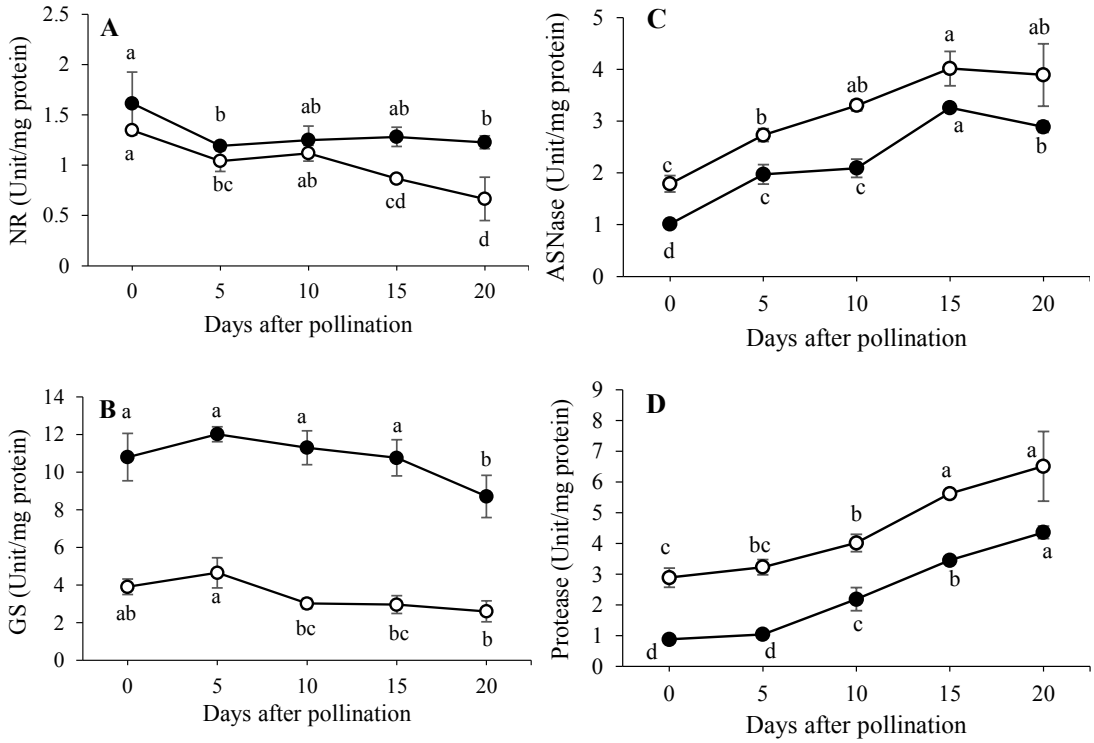

Fig. 5. Enzymes: Nitrate reductase (NR) (Unit/mg protein) (A), glutamine synthetase (GS) (Unit/mg protein) (B), asparaginase (ASNase) (Unit/mg protein) (C), and protease (Unit/mg protein) (D) of ear leaf from 0 DAP to 20 DAP under low N and high N treatments [Shown are the means of three biological replicates and error bars represent ± SD. Different letters indicate significant differences among samples within each N treatment at P<0.05. Closed symbols correspond to high N and opened symbols correspond to low N]

Discussion

The current study provides a picture connecting maize grain yield and source:sink relationships to the progressive metabolic changes associated with the gradual transition of ear leaf from assimilation to remobilization during grain filling under sufficient and deficient N fertilization. N-deficient Tzi8 x Mo17 plants yielded 43 % less grains and such reduction was associated with 26% reduction in kernels number per ear (Table 1). In agreement with our results, Liu et al. (2018) and Ning et al. (2018b) reported similar suppressive effect of N limitation on maize grain yield and its components. Such low N-induced drops in yield and kernel number is attributed to the responsiveness of Tzi8 x Mo17 plants to N availability and the biological significance of N for grain yield. N limitation reduces post-silking biomass accumulation (Table 2) which initiates a series of events including reduction in biomass allocation to ear (Fig. 1) and higher rate of kernel abortion which consequently explain the obtained reduction in kernel number, lower SSR, and grain yield (Ciampitti & Vyn, 2012; Chen et al., 2016).

Although N-deficient plants accumulated less dry matter in their leaves, stem, and developing ear (Table 2), they accumulated higher biomass in root and increased their R/S ratio at silking compared to N-sufficient plants (Table 3). These results indicate that N-deficient Tzi8 × Mo17 plants invest more of their resources in roots to improve their ability to absorb more soil N. In contrast, N-sufficient plants invest more resources in leaves and stem to maximize the capture for aboveground resources. Our results are thus consistent with the balanced-growth hypothesis and previous reports which indicate preferential biomass allocation to plant organ that increases the capture of the limiting external resource (Shipley & Meziane, 2002; Chen et al., 2013; Yu et al., 2015). Therefore, N-deficient plants had higher NUpE, NUtE, and NUE (Table 3) suggesting that such differential biomass allocation strategies contribute significantly to the improved NUE (Yu et al., 2015).

TABLE 5. Pearson correlation coefficients (r) between ear leaf metabolites across N regimes and sampling dates

	Chlorophyll	Sucrose	TSS	starch	TSP	Nitrate	Ammonia	Leaf N	NR	GS	ASNase	Protease	Anthoc-yaninis
Sucrose	0.48												
TSS	0.46	0.88***											
starch	-0.52*	0.31	0.09										
TSP	0.90***	0.66**	0.72**	-0.46									
Nitrate	0.87***	0.16	0.20	-0.74**	0.75***								
Ammonia	0.85***	0.66**	0.64**	-0.31	0.85***	0.80***							
Leaf N	0.91***	0.62**	0.55*	-0.35	0.86***	0.83***	0.96***						
NR	0.80***	0.23	0.15	-0.48	0.62***	0.89***	0.81***	0.81***					
GS	0.76***	0.76***	0.75**	-0.24	0.85***	0.58***	0.86***	0.84***	0.56				
ASNase	-0.81***	-0.10	-0.13	0.76***	-0.72***	-0.91***	-0.68***	-0.73***	-0.81***	-0.48			
Protease	-0.93***	-0.57*	-0.57*	0.50	-0.93***	-0.88***	-0.93***	-0.93***	-0.83***	-0.79***	0.84***		
Anthocyanins	-0.74***	-0.13	-0.17	0.61*	-0.63***	-0.89***	-0.72***	-0.74***	-0.84***	-0.50***	0.71**	0.75***	
Flavonoids	-0.86***	-0.17	-0.22	0.79***	-0.81***	-0.95***	-0.69**	-0.75**	-0.79***	-0.56*	0.95***	0.87***	0.79***

*Significant at P< 0.05, ** Significant at P< 0.01, *** Significant at P< 0.001.

Analysis of metabolic interconversions underlying the differential responses in yield and post-silking biomass accumulation under limited and sufficient N fertilization revealed earlier chlorophyll degradation (Fig. 2A), consistently lower levels of sucrose (Fig. 2B), and TSS (Fig. 2C) but higher starch accumulation in N-deficient than N-sufficient plants (Fig. 2D). Consistent with the current results, Hirel et al. (2005b) and Peng et al. (2016) reported inhibitory effects of N limitation on chlorophyll and various carbohydrates fractions during grain filling. These results are ascribed to the limited sink size in N-deficient plants (Table 1) and indicate differences in the dynamic of C assimilation under limited and adequate N conditions during grain filling (Zinselmeier et al., 1995). The early initiation of chlorophyll catabolism along with the concomitant decline in sucrose and TSS highlight the early onset and progression of leaf senescence as well as earlier transition of leaf physiology from assimilation to remobilization under limited N supply (Lim et al., 2007).

N-deficient and N-sufficient plants exhibited continuous and temporary leaf starch accumulation, respectively (Fig. 2D). Such patterns of starch accumulation is attributed mainly to comparable continuous and temporal sink limitation which favors sugar-to-starch conversion over sugar transport via inhibition of sucrose transport from leaves to grains (Peng et al., 2014; Ning et al., 2018b). In addition, in N-deficient plants, N limitation hampers the ability of kernels to utilize C assimilates via inhibition of kernel invertase activity (Below et al., 2000) which may exaggerate starch accumulation in source leaves. In agreement with our findings, positive correlations between source-sink ratio and leaf starch accumulation have been reported in many source-sink manipulation studies (Kasai, 2008; Iglesias et al., 2002). Further, induction of starch-synthesizing as well as inhibition of starch-degrading enzymes was reported in N-deficient rice before heading (Ishimaru et al., 2007; Li et al., 2018). Our results thus support the physiological significance of starch as a temporal storage for photoassimilates and as an effective regulatory node of source-sink relations until the growing kernels build up enough sink capacity to accommodate photoassimilates. The limited sink-induced alterations in C metabolism in N-deficient Tzi8 x Mo17 plants (earlier chlorophyll degradation, reduced sugars and accumulation of starch) were associated with accumulation of total anthocyanins (Fig. 3A) and total flavonoids (Fig. 3B) as senescence progressed. The induction of these secondary metabolites could be attributed to the low N- induced upregulation of flavonoid biosynthetic genes such as "PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1)", and PAP2 (Sekhon et al., 2012). Our results thus support similarities in senescence induced by N deficiency and pollination prevention where absence of efficient sink in both treatments results in failure of efficient carbon export to ear (Sekhon et al., 2012).

Compared to sufficient N treatment, N-deficient Tiz8 x Mo17 plants had general reductions in leaf N (23%), nitrate (40%), ammonia (41%) as well as total and individual amino acids (Fig. 4 & Table 4). These results indicate significant alterations in N metabolism in N-deficient plants which could be attributed to the limited N supply and the reduced capacity of nitrate transport from roots. The reduced levels of leaf N and other N fractions lead to reduction in leaf TSP (Fig 4A) which eventually result in overall reduction in physiological interplay in leaves since TSP is the driving force for the physiological processes (Hirel et al., 2005a; El-Kereamy et al., 2011). Regardless the reduced levels of N metabolism related biomarkers under limited N input, N-deficient and N-sufficient plants maintained similar progressive decline in the leaf N, ammonia, nitrate, and proteins as grain filling proceeds with the former being consistently lower than the later (Fig. 4). These results highlight a common shift of leaf N metabolism from assimilation to remobilization which occurs earlier in N-deficient plants (Hirel et al., 2005a; Uribelarrea et al., 2009). The significant reduction in amino acids in N-deficient plants (Table 4) is a logic outcome to the above reduction in N metabolism biomarkers and is ascribed to the reduced level of N assimilation and anabolic activity of leaf under N limitation. Interestingly, both N-deficient and N-sufficient plants also maintained relatively similar percentages of glutamate, aspartate and the branched chain amino acids (leucine & valine) in their amino acid pole at pollination and 10 DAP. These results nicely fit with the biological significance of glutamate in maintaining N homeostasis and as an important link between N and C metabolisms (Seebauer et al., 2004; Forde & Lea, 2007) and aspartate as a transitory store of N required for ear growth (Lee & Tollenaar, 2007) as well as leucine and valine

as precursors for energy-associated metabolites and signaling molecules (Pratelli & Pilot, 2014; Tatjana et al., 2015). Altogether, such response represents an additional common node between N-sufficient and N-deficient plants' physiology during leaf transition from assimilation to remobilization. N-deficient plants also had reduced level of leaf serine which is a marker for induced photorespiration and senescence (Tercé-Laforgue et al., 2004: Diaz et al., 2005) as has been reported in sunflower (Agüera et al., 2010) and wheat (Heyneke et al., 2019).

Our enzyme analysis revealed strong positive correlations between N assimilation enzymes (NR & GS) and leaf chlorophyll, total N and all N fractions (Table 5). These results suggest that the two enzymes, particularly GS, is an excellent indicator for N status of maize plants as well as N transport during remobilization. Consistent with this view, N-sufficient plants consistently had higher NR and GS activity than N-deficient plants; however, the difference in GS was always higher than NR at all stages of grain filling (Fig. 5A & B). In support of the current results, Masclaux et al., (2000) and Kaur et al. (2019) reported down regulation of primary N assimilation enzymes as leaf ages in response to N limitation. The low N-induced reduction in NR and GS activities is attributed to the reduced fertilizer input and its physiological consequences on the levels of leaf N, nitrate and ammonia (Hirel et al., 2001; White et al., 2016). Also, Latif et al. (2021) obtained similar reduction in GS activity under other abiotic stress such as salinity in barely. The reduced activities of NR and GS explain the reduction in amino acids synthesis in the ear leaf of N-deficient plants compared to N-sufficient plants (Table 4). The relatively similar dynamics of NR, GS, their substrates (nitrate& ammonia), and the other fractions (leaf N & TSP), thus suggest a coordinated regulation of the physiological interconversions of these metabolites (Stitt, 1999). They also support the role of GS as a marker of both inorganic N assimilation and recycling (Masclaux et al., 2000; Li et al., 2016) as well as one of the main checkpoints controlling the plant N status, kernel set, and grain filling (Yu et al., 2016).

In contrast to the down regulation of NR and GS, our analysis revealed a progressive increase in protease and ASNase activity in both N-deficient and N-sufficient plants as grain filling progressed (Fig. 5C & D). Such common induction is consistent with their catabolic roles during resource remobilization (Zimmermann & Zentgraf, 2005). The consistently higher activities of ASNase and protease activity in N-deficient plants indicate greater metabolic degradation and remobilization under low N input. Consistent with the current results, Schlüter et al. (2012) reported the induction of ASNase transcripts in maize source leaves under N limiation. In addition, a transposon-induced mutation in a maize leaf protease retarded the low–N induced decline in senescence-related resource remobilization and induced a "delayed senescence phenotype" in the mutants (Donnison et al., 2007). Induction of protease activity (Fig. 5D) was coordinated with chlorophyll degradation (Fig. 2A), decline in TSP (Fig. 4D), and increased leaf N remobilization (Fig. 4A). It also showed a strong negative correlation with all measured metabolites except starch, anthocyanins and flavonoids (Table 5). These results thus indicate the regulatory role of protease in most of the catabolic events in ear leaf during grain filling (Smart et al., 1995; Donnison et al., 2007). The elevated activity of ASNase may be required to hydrolyze asparagine accumulated in response to N stress (Lea & Miflin, 2011) and/or that released from protein catabolism in leaf during grain filling.

Conclusion

During leaf transition from assimilation to remobilization, the ear leaf of N-deficient and N-sufficient maize plants are programmed to maintain common patterns of dynamics of most of the analyzed carbon and N metabolites. Yet, N-deficient plants specifically activate signaling cascade(s) that derives higher biomass allocation to roots to maximize N acquisition and higher starch accumulation as well as earlier senescence events such as chlorophyll degradation, higher activities of N-remobilization enzymes (protease and ASNase), and reduced serine levels. In addition, N-deficient plants specifically accumulate falvonoid compounds particulary at latter stages which seems to be an important node in the signalling caccade (s) driving resourse remolization during grain filling. Further, limited N leads to premature depletion of ear leaf N which in turn leads to failure to fully export leaf C to the developing ear. Such responses, along with the low N-induced sink limitation, contributed significantly to the reduced growth and yield under low N input.

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Authors contribution: El-Shahaby OA, Abo-Hamed SA and Ibraheem F designed and supervised the work. Elghareeb EM carried out the experiments, data acquisition and statistical analysis. All authors interpreted the data. Elghareeb EM and Ibraheem F wrote the first draft of the work. All authors revised the draft critically and approved the final version to be published.

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الإستجابات الأيضية لمستويات متضادة من التسميد النيتروجيني أثناء تحول ورقة الكوز من أيض البناء إلى أيض الهدم أثناء ملئ الحبوب في الذرة

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تعتمد انتاجية نبات الذرة على محصلة التأثيرات الأيضية المتبادلة بين الأنسجة التمثيلية والأنسجة المستقبلة لنواتج الأيض خلال مرحلة ملء الحبوب. هذه التأثيرات الأيضية ذات طبيعة مركبة وتتأثر كثيرا بمستوى نيتروجين التربة وما زالت غامضة إلى حد كبير وخاصه في الهجن الجديدة. لذلك تم دراسة تأثير مستويات متضادة (منخفضة وعالية) من التسميد النيتروجيني على التحولات الأيضية التي تحدث في ورقة الكوز لهجين الذرة (Tzi8×Mo17) والذي انتجناه حديثا والنامي في الحقل عند فترات زمنية متتابعة (0، 5، 10، 15، 20 يوم بعد التلقيح) أثناء التحول التدريجي للورقة من طور الأيض البنائي إلى أيض الهدم ونقل المركبات الأيضية إلى الحبوب. أدى نقص النيتروجين إلى انخفاض بنسبة 43% في انتاج الحبوب و13% في الوزن الجاف للمجموع الخضري في مقابل زيادة الوزن الجاف للجذر بنسبة 51%. كان ذلك مصحوبا بنقص في الكلوروفيل والبروتينات والسكروز والسكريات الذائبة وكذلك صور النيتروجين مثل النيتروجين الكلي للورقة والنترات والأمونيا والبروتينات الكلية الذائبة بالإضافة إلى تقليل نشاط انزيمي nitrate reductase وglutamine synthetase بالمقارنة مع زيادة النيتروجين. على النقيض سبب نقص النيتروجين زيادة في محتوى الأوراق من النشا ومركبات الفلافونويد وزيادة نشاط إنزيمي protease وasparaginase . توضح النتائج أن أوراق الذرة مهيئة بأنها تفعل انماط أيضية خاصة تحت ظروف نقص النيتروجين في مقابل المحافظة على أنماط أيضية أخري مشتركة بين ظروف التسميد النيتروجيني العالي والمنخفض.