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Impact of Nitrogen Availability on the Accumulation of Vegetative Lipids in Sorghum

Leticia Felicio Pasqualino
University of Nebraska - Lincoln

IMPACT OF NITROGEN AVAILABILITY ON THE ACCUMULATION OF VEGETATIVE LIPIDS IN SORGHUM

by

Letícia Felicio Pasqualino

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IMPACT OF NITROGEN AVAILABILITY ON THE ACCUMULATION OF VEGETATIVE LIPIDS IN SORGHUM

Leticia Felicio Pasqualino, M.S.

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Advisors: Thomas Clemente and Jeffrey Mower

Sorghum (*Sorghum bicolor* (L.) Moench) is a versatile and prosperous feedstock crop for renewable energy production. With the increase in bioenergy demand, the production of higher oil content per biomass in a feedstock crop is a highly desirable trait for the biofuel conversion process yield. Especially if this outcome can be achieved using fewer inputs in the field, such as nitrogen. In microalgae species, nitrogen limitation has been associated with changes in the carbon storage metabolic pathway favoring triacylglycerol (TAG) accumulation. In this context, this study aimed to assess whether nitrogen starvation would result in a similar outcome in higher plants. Nitrogen limitation's impact on sorghum vegetative lipids accumulation was investigated in two sorghum genotypes. In a soilless trial, greenhouse-grown plants were submitted to three levels of nitrogen supply: 0.0 mM, 0.75 mM, and 15 mM KNO_3 delivered in nitrogen-free Hoagland's liquid solution. Total fatty acids (TFA), TAG and biomass were analyzed in three developmental stages (7-leaf, flowering, and post-flowering). At the 7-leaf stage, lower nitrogen availability resulted in significantly higher TAG content, where the values for 0.0, 0.75, and 15 mM N treatments were 0.97, 0.72, and 0.44 TAG %DW, respectively (P=0.04). The same results pattern was observed at the flowering stage, where lower nitrogen resulted in 1.24 TAG %DW, while

higher nitrogen resulted in 0.78 TAG %DW. Post-flowering TAG content did not present a significant difference across nitrogen treatments. The TFA content increased with nitrogen availability, and the nitrogen treatment effect was significant at the flowering (P<0.01) and post-flowering (P=0.02) stages. Across all stages, biomass was significantly higher with increased nitrogen availability. The research findings are initial steps in comprehending the impact of nitrogen availability on sorghum vegetative lipids and provide valuable insights for optimizing the balance of nitrogen application and biomass yield ratio to achieve valuable and sustainable sorghum production for biofuel feedstock.

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INTRODUCTION

Sorghum

Considered a versatile and sustainable multipurpose crop, *Sorghum bicolor* (L.) Moench is a C4 grass (Poaceae family) cultivated for human consumption, animal feed, fiber and biofuels. Originating in equatorial Africa, semi-arid regions, one of the main strengths of sorghum is its drought tolerance, enabling its cultivation in regions with limited water availability (Stone et al., 1996). In addition to being more efficient in photosynthesis and nitrogen-based fertilizer use, as a C4 grass, sorghum can also be cultivated in marginal lands that are not suitable for food production. This makes sorghum a relatively low-input crop and a valuable option for maximizing productivity in challenging agricultural environments and future climate conditions (Hossain et al., 2022).

With that being said, sorghum is also a promising crop for biofuel production due to its high biomass yield, drought tolerance and adaptability. It has the potential to contribute to renewable energy and reduce dependence on fossil fuels while not competing with food production.

Being the third most cultivated grain in the United States (US), the USDA (2023) reports that the annual global production of sorghum reached 62,180 thousand metric tons (MT). The US is the leading producer, producing 11,375 thousand MT and contributing 18% to global production, followed by Nigeria (11%) and Mexico (8%). Within the US,

the primary producer states are Kansas, responsible for 55% of the country's production, and Texas, accounting for 21%. It’s important to notice that the US had nearly twice the production of the third-ranked producer.

Biofuels

Biofuels are an alternative to fossil fuels due to their potential to minimize greenhouse gas emissions and the reliance on non-renewable energy sources. These fuels can be derived from various organic matter sources: agricultural and energy crops, agricultural residues and algae. For biofuel feedstocks, it is important to prioritize those that do not compete with food production and have lower impacts on the environment. Recent advancements in conversion technologies have improved efficiency and increased the availability of feedstocks (Jung et al., 2021; Liu et al., 2021), which have expanded the potential for biofuel production worldwide.

Additionally, environmental concerns and policies promoting renewable energy have increased the demand for biofuels (OECD-FAO, 2022). Energy security, reduction of greenhouse gas emissions, and renewable energy policies encourage the adoption of biofuels in transportation sectors. Therefore, further research is necessary to enhance the overall capacity and sustainability of its production.

Biofuel is the broad term used for all diverse types of fuel derived from renewable energy sources, where the two most common are ethanol and biodiesel. Most ethanol is made from plant starches and sugars, such as sugarcane and corn, the last being the most

commonly used source in the US. Biodiesel is a liquid fuel produced from renewable sources, such as new and used vegetable oils and animal fats (Demirbas, 2008).

Sorghum can be converted into various types of biofuel, including ethanol and biodiesel, through different conversion processes (Dar et al., 2018). Ethanol is the most commonly produced biofuel from sorghum. However, when compared to ethanol, biodiesel has a higher energy production. Despite this advantage, the use of biodiesel is constrained due to the limited supply of feedstocks. Therefore, in order to meet a significant portion of both current and future fuel needs, improvements to biodiesel feedstocks need to be achieved, such as the increase in plant lipids accumulation (Durrett et al., 2008).

Nitrogen Starvation

Due to their potential use for biodiesel production, strategies to increase lipids accumulation have been investigated in microalgae. Studies show that nitrogen starvation could be an effective strategy for boosting lipid accumulation in microalgae species. When microalgae are starved of nitrogen, there is a change in the cellular carbon flux, prioritizing carbon metabolism over protein synthesis, which leads to an increase in the production and accumulation of lipids, while biomass yield is reduced (Mujtaba et al., 2012; Yang et al., 2013; Gonçalves et al., 2016; Yaakob et al., 2021).

Arguelles & Martinez-Goss (2021) presented that limitation in nitrogen availability caused an increase in the lipid yield of microalgae species *Chlorolobion* sp. and *Chlorella* sp. Numerous studies have found similar results in various species of microalgae (Converti et al., 2009; Chen et al., 2011; Taleb et al., 2017; Shaikh et al., 2019; Andeden et al., 2021).

Study Objectives

In pursuit of a higher accumulation of vegetative lipids per plant, the presented study aimed to evaluate the impact of nitrogen availability on the levels of triacylglycerol and total fatty acids in the vegetative tissues of grain sorghum under controlled conditions, as well as in biomass yield and lipid droplets content.

MATERIALS AND METHODS

Plant Material

Two genotypes of *Sorghum bicolor* (L.) Moench were investigated. Wild-type Tx430 (WT) and TAG1 – genetically enhanced transgenic plants for higher oil content in stems and leaves.

Greenhouse Growing Conditions and Experimental Design

Soilless greenhouse studies were conducted at the University of Nebraska-Lincoln (UNL) Beadle Center Greenhouses (Institute of Agriculture and Natural Resources Plant Growth Facilities) in Lincoln, NE, USA.

The experimental design was a randomized completed block with three replications, including the two genotypes and three nitrogen treatments. The sorghum plants were submitted to three levels of nitrogen (0.0 mM, 0.75 mM and 15.0 mM KNO_3). The KNO_3 was delivered through irrigation with Hoagland's nitrogen-free solution (Hoagland Medium Nitrogen-free diluted in water - PlantMedia™, Genelinx International, Dublin, OH, USA). The experiment was conducted twice, and the rounds consisted of a total of fifty-four plants. There were two rounds in total, labeled as Round I and Round II.

For each round, approximately 35 seeds were initially sowed for each genotype, WT and transgenic. One pea pot containing growing medium Pro-Mix BX (Premier Tech Horticulture, Riviere-du-Loup, Quebec, CA) was used for each seed. The pots were placed

in the greenhouse at temperatures averaging 28 and 20°C, day and night, respectively, with a photoperiod of 12 hours (supplemental lights). No fertilization was offered at this stage.

Following the genotyping of transgenics and confirmation of transgenes presence, 27 seedlings for each genotype were transplanted to 23 by 26 cm nursery pots (C1000, Nursery Supplies, Orange, CA, USA) containing 60% vermiculite, 20% sand and 20% cultivation professional mix, Metro Mix 200 (Sun Gro® Horticulture, Agawam, MA, USA). Transplanted plants were returned to the greenhouse under the same previous environmental conditions mentioned. For experiment repetitions I and II, the replicates were arranged following the bench design shown in Figures X and Y, respectively.

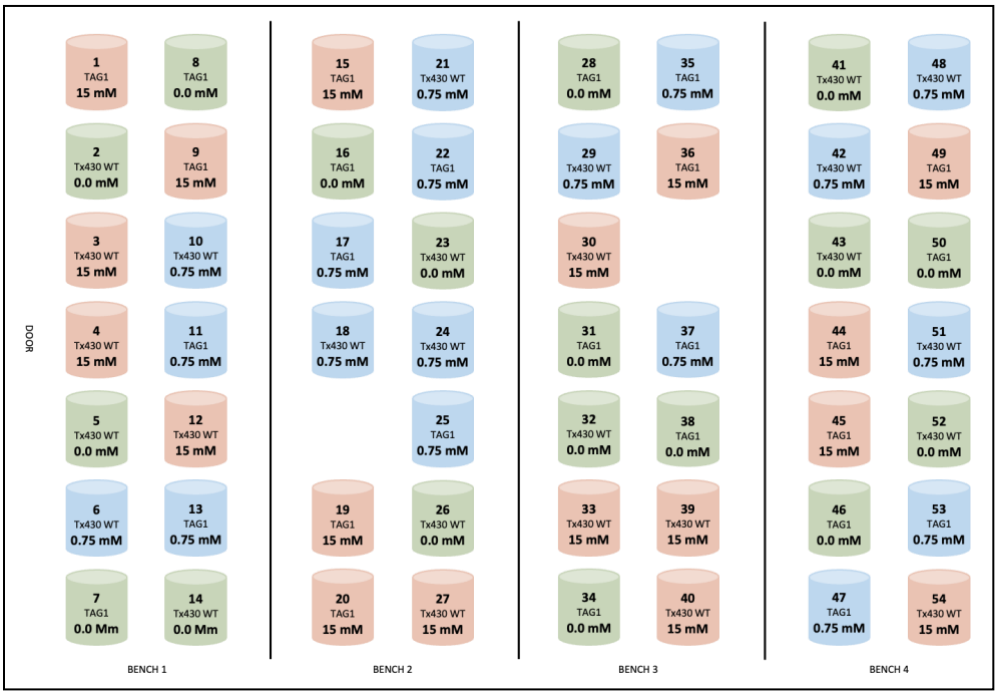

Figure 1. Biological replicates allocation in the greenhouse following a randomized complete block design in Round I.

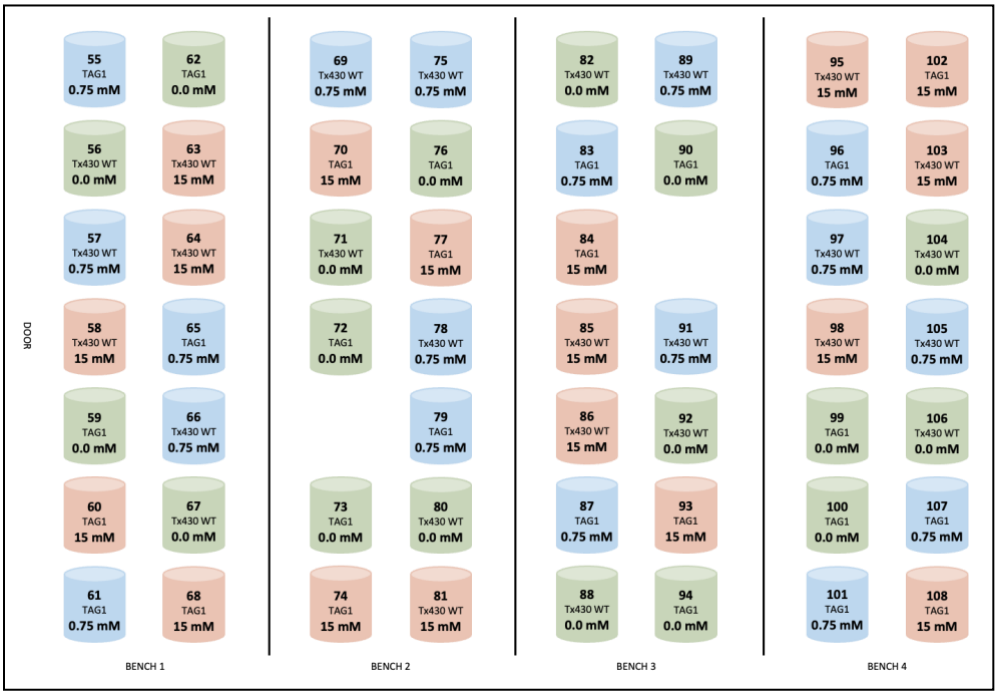

Figure 3. Biological replicates allocation in the greenhouse following a randomized complete block design in Round II.

Sample Collections

Sampling of leaves tissues for total FA and TAG analysis

Wild-type and transgenic plants were sampled for total fatty acids (FA) and TAG analysis at three different growth stages: 7-leaf stage – when the plant visibly presented the collar of the seventh fully expanded leaf, flowering – more than half of the panicle had bloomed, and post-flowering – approximately two weeks after flowering-stage samples were taken.

Leaf tissue samples from the mid-section of the first fully developed leaf were collected from eighteen plants in each developmental stage, with three biological replications per genotype and treatment combination. The samples were immediately

freeze-dried in a lyophilizer (FreeZone 12 Liter Freeze Dryer, Labconco, Kansas City, MO, USA) for 24 hours. The lyophilized tissues were used immediately for lipids extraction.

Sampling of leaves tissues for Total Nitrogen

Following the same pattern of three biological replications per genotype × treatment combination per developmental stage, leaf samples for total nitrogen quantification were collected from eighteen plants at the post-flowering stage, with the final measurements before plants were destroyed. Approximately 6 g of fresh plant tissues were sampled from a pool of all fully developed leaves. The samples were immediately submitted for analysis to Ward Laboratories Inc. (Kearney, NE).

Sampling of leaves tissues for Lipid Droplets Imaging

The same eighteen plants selected for lipids analysis at each growth stage were used for the collection of leaf tissue for microscopy imaging of lipids droplets. Sections of 1x1 cm were cut from the mid-section of the second fully developed leaf, placed in tubes with distilled water and immediately taken to Morrison Microscopy Core Research Facility at the UNL Nebraska Center for Biotechnology (Lincoln, NE). At the final sampling, 1x1 cm sections of the stem nearby the second fully developed leaf were also collected.

Lipids Extraction and Quantification

Lipids Extraction

From the lyophilized leaf tissues, 30 mg was used for lipid extraction. Based on the Bligh and Dyer (1959) method, 3 ml of methanol/chloroform solution (2:1, v/v) was added

to the plant material. Additionally, on each sample, 20 mg of triheptadecanoin (Nu-Chek Prep, Elysian, MN) dissolved in toluene was added as a TAG standard reference for future calculation of lipids values. The samples were incubated overnight on a shaker at RT. For phase separation, 1 ml of chloroform and 1.9 ml of distilled water were added to the tube, mixed by vertexing, and centrifuged for 4 min at RT. The organic phase was collected and evaporated under nitrogen gas flow at 50 °C.

A total of 120 µl of heptane was added to the dried lipid phase. The mixture was divided into three parts of 40 µl for the respective procedures: total FA analysis, TAG analysis and TLC plate staining for TAG visualization. The portion selected for total FA was re-evaporated under nitrogen gas flow at 50 °C for later transesterification.

For TAG extraction, 40 µl of lipid extract was developed on a TLC plate (Silica Gel 60, 20x20 cm, Supelco, Bellefonte, PA, USA) using a solvent system of heptane/diethyl ether/acetic acid (70:30:1, v/v/v). Crude soybean oil extract was used as a TAG running marker. The TLC plate was dried after developing, and 0.01% primuline (dissolved in 80% acetone, w/v - Sigma-Aldrich, St. Louis, MO, USA) was sprayed on the plate for TAG visualization under UV light. Based on the soybean marker, the boundaries of TAG were marked, and the region was scraped from the plate and transferred to a glass tube for later transesterification.

With minimal alterations, the previously outlined development process was used for TLC plate staining for TAG visualization. The plate was developed, dried, and then placed overnight at RT in a glass chamber with solid iodine crystals (Sigma-Aldrich, St. Louis, MO, USA). Photos of the lipids spot stains were taken for the record.

Transesterification

The dried total FA supernatant and scraped TAG were methylated using 2 ml of 2.5% sulfuric acid in methanol for 90 min at 90 °C. Tubes were cooled down at room temperature (RT) before adding 1 ml of water and heptane, 600 µl for total FA and 300 µl for TAG. After centrifugation and separation of phases at RT, the upper phase was transferred into a GC vial. Polyspring vial inserts were used for the TAG samples.

Gas Chromatography Analysis

Transesterified total FA and TAG samples were then analyzed using an Agilent Technologies 6890N network gas chromatograph (GC) system with a 60 m × 0.25 mm × 0.25 µm J&W GC column (Agilent Technologies, Santa Clara, CA, USA).

The GC oven's initial temperature was programmed to 90°C and held for 1 min, followed by an increase of 30°C/min until the final temperature of 235°C, which was maintained for 5 min. Flame ionization was used for detection (FID). Fatty acid methyl esters (FAME) peaks were recorded and identified using internal standard concentrations of fatty acids compounds retention times.

Biomass Measurements

Aboveground plant biomass was measured two weeks after flowering, following the last collection of leaf tissue samples. Fresh weight was measured as the weight of all aboveground parts, totaling two final values – including and not including grains. Fresh biomass was put in a convection oven at 65°C for 48 h for the obtention of dry weights.

Lipid Droplets Imaging by Confocal Microscopy

The fresh leaf and stem sections were thin hand-cut and stained for 5 min with a solution of 2 μg/ml Nile Red (Thermo Fisher Scientific, Carlsbad, CA, USA) in distilled water. The stained sections were mounted on the slides, and the chloroplasts were used to establish the background fluorescence of the tissue.

Confocal imaging of samples was performed on a Nikon A1 Confocal Laser-Scanning Microscope with an upright Ni-E fluorescent microscope. Confocal image data acquisition was acquired with Nikon NIS-Elements.

The red channel (Nile Red) was excited using a laser line with a wavelength of 561 nm, and the green channel (chloroplasts) was excited using a laser line with a wavelength of 640 nm. Imaging at 20x magnification was performed using a Nikon Plan Apo 20x/0.75 WD 0.17 mm lens, and for imaging at a 60x, Nikon Plan Apo VC 60xA/1.20 WI with a WD ranging from 0.15 to 0.18 mm was utilized.

Photosynthesis and Chlorophyll Measurements

An LI-6400 XT Portable Photosynthesis Analysis System (LI-COR, Lincoln, NE, USA) was used to measure stomatal conductance, intercellular CO_2 concentration, transpiration rate, and consequent photosynthetic rate of the sorghum leaves. The data was collected between 11:00 and 14:00 on three clear and cloudless days at the flowering growth stage. For each biological replicate, two random measurements were taken from the top layer of leaves. The light source's effective radiation was PAR 1200 μmol $m^{-2}s^{-1}$, the leaf chamber used was 2×3 cm, and the fixed flow rate was set at 500 μmol s^{-1}, while

sample CO_2 was controlled at 400 µmol s^{-1}. The relative humidity was manually kept at 50% or greater.

Chlorophyll content was measured using a SPAD 502 Plus Chlorophyll Meter (Spectrum Technologies, Aurora, IL, USA) also at the flowering stage. Numerical values (SPAD), proportional to the leaf chlorophyll content, were obtained by the average of two sets of measurements from 3 section points per biological replicate. Leaves used for measurements were randomly selected from the mature leaves in the canopy.

Statistical Analysis

All the data were analyzed as a two-way factorial in PROC GLIMMIX (SAS Studio, version 3.81, SAS Institute 2020) to determine the significance of nitrogen treatment, genotype and nitrogen × genotype interactions. In PROC GLM, the grouping of nitrogen treatment was defined by Tukey's Studentized Range Test. The effects were considered significant when $P \leq 0.05$. GraphPad Prism 9 software was used for graphs.

RESULTS

Vegetative Lipids

Table 1 presents the results of the vegetative lipids for both genotypes and the two rounds of experiments combined. The average of the triacylglycerol (TAG) content exhibited a significant decrease with increased nitrogen (N) availability at the 7-leaf stage from 0.97 at 0.0 mM to 0.44 %DW at 15.0 mM N treatment with P=0.04. However, no significance was observed at the flowering stage for the decrease of 1.24 %DW at 0.0 mM to 0.78 %DW at 15.0 mM KNO_3 (P=0.32). At the post-flowering stage, TAG did not show significant differences across N treatments (P=0.99), presenting 0.82, 1.16 and 1.20 %DW TAG for 0.0, 0.75 and 15.0 mM N treatments, respectively.

Across all developmental stages, total fatty acids (TFA) increased with higher N levels. The nitrogen treatment effect was found to be significant during the flowering (P<0.01) and post-flowering stages (P=0.03), with an increased average of 4.02 to 6.78 %DW and 4.20 to 6.57 %DW, respectively. The increase from 5.25 %DW to 6.03 %DW at the 7-leaf stage was not found to be significant (P=0.34).

There was no nitrogen × genotype interaction or block effect observed on TAG or TFA. However, a significant genotype effect was observed for TAG at all three developmental stages (P<0.01). During the flowering stage, TFA also showed a genotype effect (P<0.01), as well as during the post-flowering stage (P=0.02), but not during the 7-leaf stage (P=0.12). The genotype effects can be visualized in Figures 3 and 4.

Table 1. Impact of three levels of nitrogen availability on triacylglycerol and total fatty acids in sorghum at three developmental stages: 7-leaf, flowering, and post-flowering. Lipids are expressed as percentages per dry weight, and the values represent the combined results from two rounds of experiments for both WT and TAG1 genotypes.

Fatty Acids, %DW	Nitrogen Treatment, mM[1]			*P*-value[2]				
	0.0	0.75	15.0	R	B	N	G	N × G
				7-Leaf Stage				
TAG	0.97[a]	0.72[ab]	0.44[b]	**<.01**	0.99	**0.04**	**<.01**	0.26
Total FA	5.25[a]	5.12[a]	6.03[a]	**<.01**	0.88	0.34	0.12	0.44
				Flowering Stage				
TAG	1.24[a]	0.80[a]	0.78[a]	**0.02**	0.35	0.32	**<.01**	0.46
Total FA	4.02[a]	4.92[ab]	6.78[b]	0.06	0.20	**<.01**	**<.01**	0.76
				Post-Flowering Stage				
TAG	0.82[a]	1.16[a]	1.20[a]	**0.02**	0.71	0.99	**<.01**	0.94
Total FA	4.20[a]	5.21[ab]	6.57[b]	0.92	0.29	**0.03**	**0.02**	0.51

[1]0.0, 0.75 and 15.0 Mm KNO_3. [2]R = round effect; B = block effect; N = nitrogen effect; G = genotype effect; N x G = nitrogen × genotype interaction effect. Numbers followed by different letters represent significant differences at the $P \leq 0.05$.

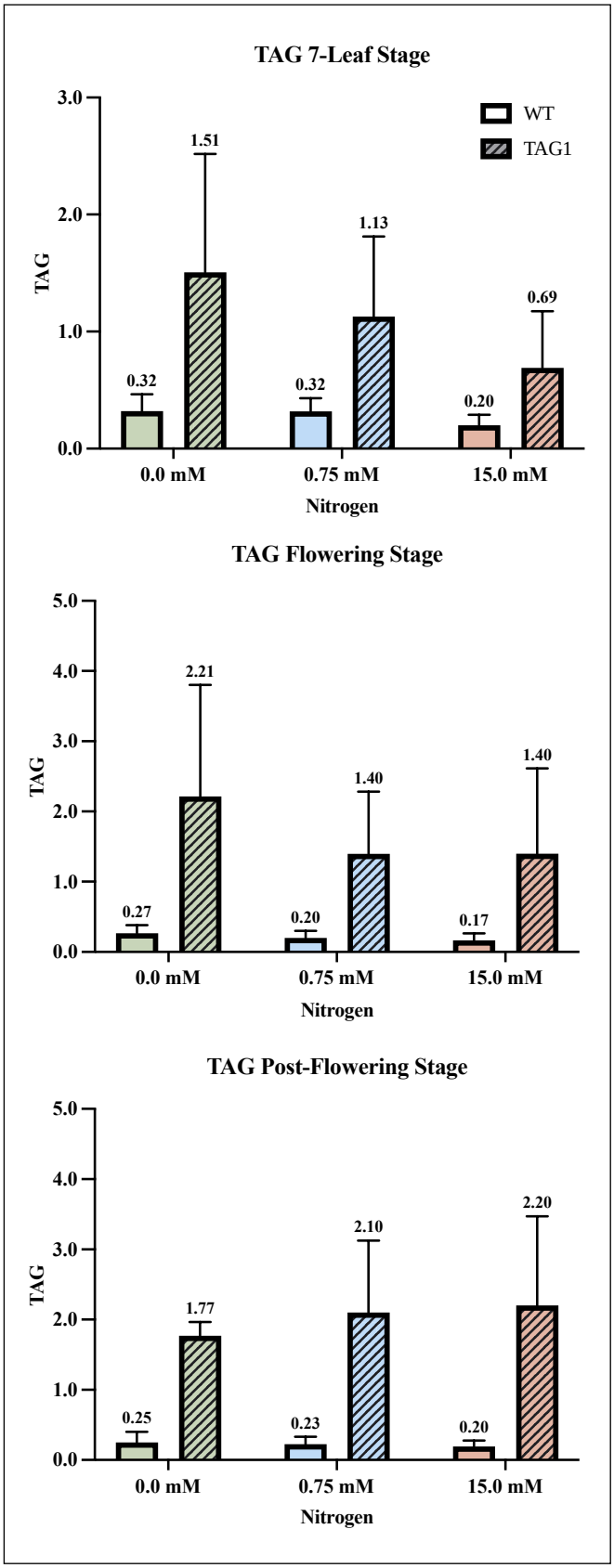

Figure 4. Interleaved bars graph illustrating triacylglycerol (TAG) values in percentage per dry weight (%DW) across three different nitrogen levels, 0.0, 0.75 and 15.0 mM KNO_3. TAG content averages for two genotypes of *Sorghum bicolor*, WT and TAG1 are displayed for 3 developmental stages: 7-leaf, flowering and post-flowering (around 2 weeks after flowering). Error bars indicate standard deviations of the means.

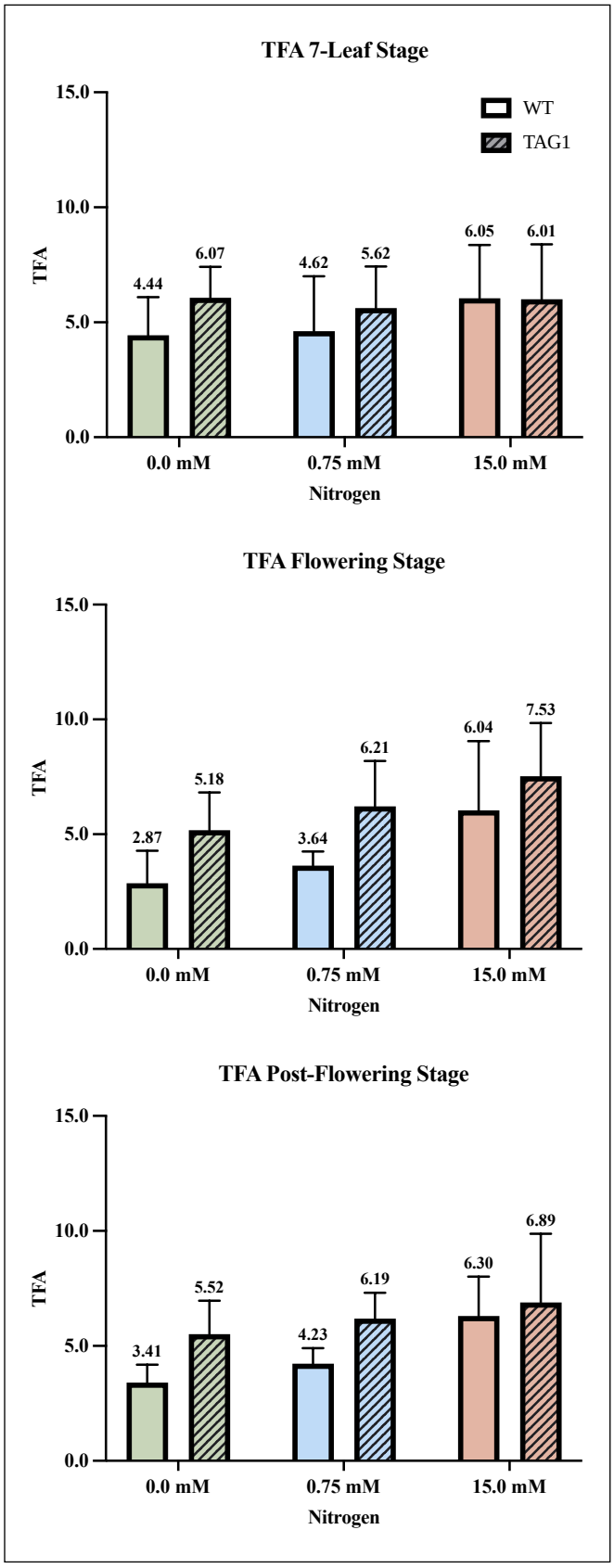

Figure 5. Interleaved bars graph illustrating total fatty acids (TFA) values in percentage per dry weight (%DW) across three different nitrogen levels, 0.0, 0.75 and 15.0 mM KNO_3. TFA content averages for two genotypes of *Sorghum bicolor*, WT and TAG1 are displayed for 3 developmental stages: 7-leaf, flowering and post-flowering (around 2 weeks after flowering). Error bars indicate standard deviations of the means.

In the 7-leaf and flowering stages, both the WT and TAG1 genotypes exhibited similar patterns, with lower values at a 15 mM N treatment and higher values at 0.0 mM N treatment for TAG. However, at the post-flowering stage, the TAG1 genotype displayed a positive correlation between TAG content and increased N availability, while the WT genotype showed a slight decrease in TAG content with an increase in N. TAG1 genotype presented higher TAG values than the WT for all cases, treatments and stages (Figure 3). For TFA, both genotypes showed an increase in total lipids with an increase in N availability across all three developmental stages. TAG1 genotype presented higher TFA values than the WT in almost all treatments and stages, with the exception of the 15.0 mM N treatment at the 7-leaf stage (Figure 4).

As shown in Table 1, a significant round effect was present at the 7-leaf stage for both TAG and TFA, as well as at the flowering and post-flowering stages for TAG. Therefore, the data were also analyzed separately for Round I and Round II of the experiments (Table 2).

As previously observed in the combined analysis, the TAG content shows a decrease as the availability of N increases during the 7-leaf and flowering stages for both Rounds I and II of experiments. This decrease is significant in Round I at the 7-leaf stage (P=0.02), where the values decrease from 1.59 %DW at 0.0 mM to 0.63 %DW at 15.0 mM KNO_3.

At the post-flowering stage, the TAG content did not consistently follow the same pattern in Rounds I and II and did not always conform to the overall trend observed in the combined analyses. In Round I, there was no plant material available for lipids extraction at 0.0 mM treatment for the TAG1 genotype, and only two biological replicates were suitable for analysis for the WT genotype. Based on the data collected, there was a non-significant increase in TAG content as N availability increased from 0.75 mM to 15 mM KNO_3, which does follow the previous trend observed. Oppositely, in Round II, a non-significant decrease in TAG content was observed with the increase of N levels (Table 2). However, to summarize, no statistically significant differences were observed in TAG in the post-flowering stages when N availability was modified.

Throughout both rounds and all three developmental stages, an increase in TFA was observed and positively correlated with an increase in N availability. However, this increase was found to be statistically significant only during the flowering stage of Round II (P=0.03). Which, on average, TFA increased from 4.91 to 7.67 %DW at 0.0 mM and 15.0 mM N treatments, respectively.

During both rounds, a significant genotype effect was observed for TAG at all three developmental stages. However, the genotype only presented a significant effect on TFA during the flowering stage of Round I and the post-flowering stage of Round II. No genotype effect was present on TFA in the remaining stages across the rounds of experiments. Additionally, there was no interaction between nitrogen and genotype or block effect observed on the separate analyses of TAG or TFA across the two rounds.

The TAG and TFA averages for each genotype and round of the experiment are displayed in Figures 5-6. In the illustrations, it's possible to observe genotype and round effects that were observed in Tables 1-2. Notably, the TAG content for the WT and TAG1 genotypes did not follow the same pattern during the flowering and post-flowering stages of Round I and the post-flowering stage of Round II (Figure 5). A slight distinction of patterns between genotypes of TFA response to the increase on N availability can be observed during both 7-leaf stages (Figure 6). However, due to the limited number of replicates and the non-significant effect of N treatment for these stages, statistical analyses were not conducted separately for each genotype to determine significance.

Table 2. Impact of three levels of nitrogen availability on triacylglycerol and total fatty acids in sorghum at three developmental stages: 7-leaf, flowering, and post-flowering. Lipids are expressed as percentages per dry weight, and the values represent the combined results of WT and TAG1 genotypes at two rounds of the experiments.

Fatty Acids, %DW	Nitrogen Treatment, mM[1]			*P*-value[2]				Nitrogen Treatment, mM[1]			*P*-value[2]			
	0.0	0.75	15.0	B	N	G	N x G	0.0	0.75	15.0	B	N	G	N × G
	Round I							**Round II**						
	7-Leaf Stage													
TAG	1.59^{a}	0.99^{ab}	0.63^{b}	0.91	**0.02**	**<.01**	0.05	0.44^{a}	0.45^{a}	0.26^{a}	0.96	0.28	**0.02**	0.84
Total FA	4.13^{a}	4.06^{a}	4.57^{a}	0.93	0.90	0.10	0.60	6.37^{a}	6.17^{a}	7.48^{a}	0.95	0.12	0.86	0.38
	Flowering Stage													
TAG	1.68^{a}	0.99^{a}	1.16^{a}	0.39	0.52	**<.01**	0.62	0.80^{a}	0.60^{a}	0.41^{a}	0.49	0.13	**<.01**	0.26
Total FA	3.13^{a}	4.87^{a}	5.89^{a}	0.37	0.07	**<.01**	0.89	4.91^{a}	4.98^{ab}	7.67^{b}	0.40	**0.03**	0.29	0.32
	Post Flowering Stage													
TAG	-	1.44^{a}	1.69^{a}	0.48	0.82	**<.01**	0.40	1.00^{a}	0.89^{a}	0.71^{a}	0.92	0.28	**<.01**	0.23
Total FA	-	5.50^{a}	6.41^{a}	0.06	0.14	0.41	0.37	4.69^{a}	4.93^{a}	6.69^{a}	0.79	0.06	**0.05**	0.76

[1]0.0, 0.75 and 15.0 Mm KNO_3. [2]B = block effect; N = nitrogen effect; G = genotype effect; N x G = nitrogen × genotype interaction effect. Numbers followed by different letters represent significant differences at the $P \leq 0.05$.

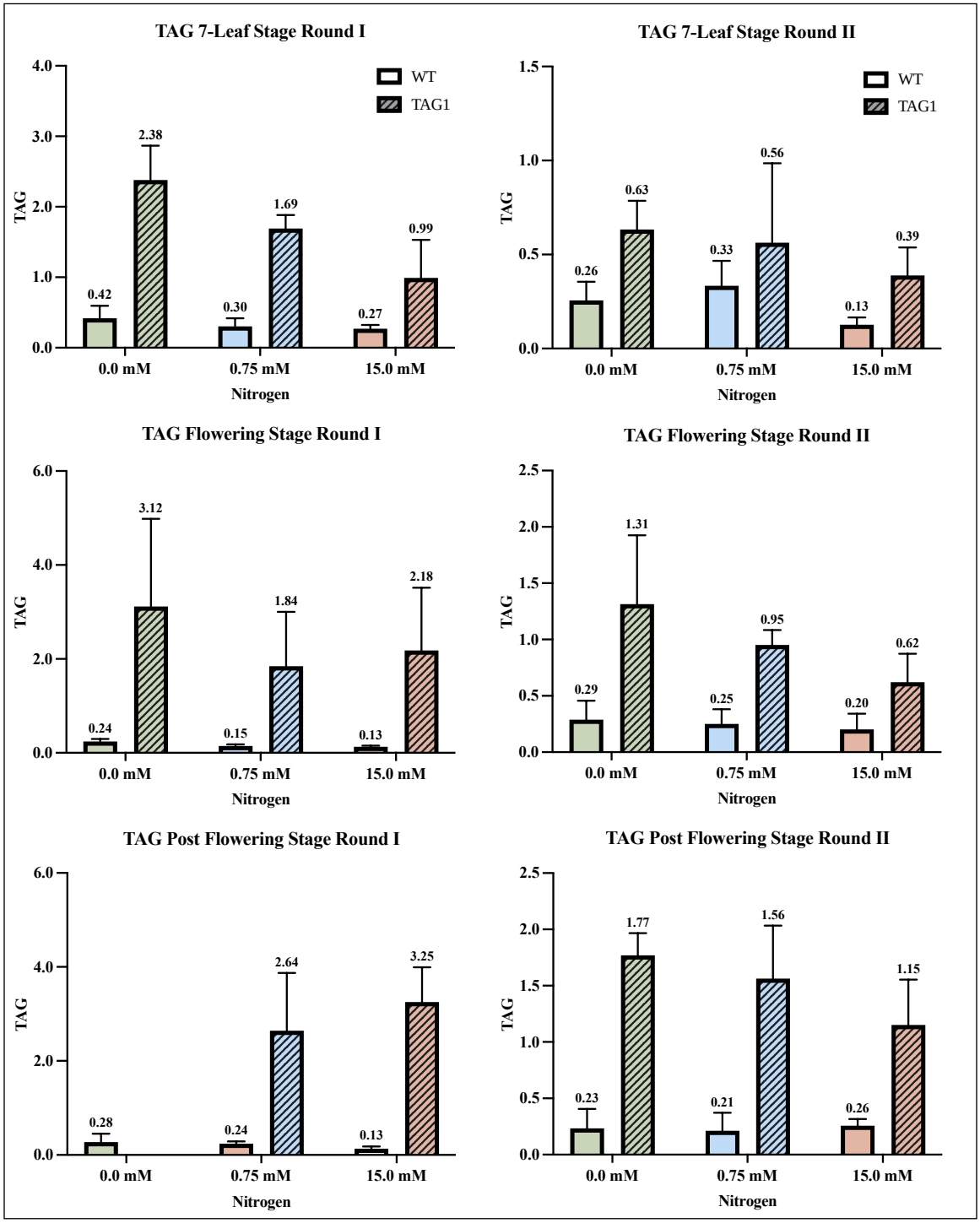

Figure 6. Interleaved bars graph illustrating triacylglycerol (TAG) values in percentage per dry weight (%DW) across three different nitrogen levels, 0.0, 0.75 and 15.0 mM KNO_3. TAG content averages for two genotypes of *Sorghum bicolor*, WT and TAG1, are displayed for 2 rounds of the experiments separated and 3 developmental stages: 7-leaf, flowering and post-flowering (around 2 weeks after flowering). Error bars indicate standard deviations of the means.

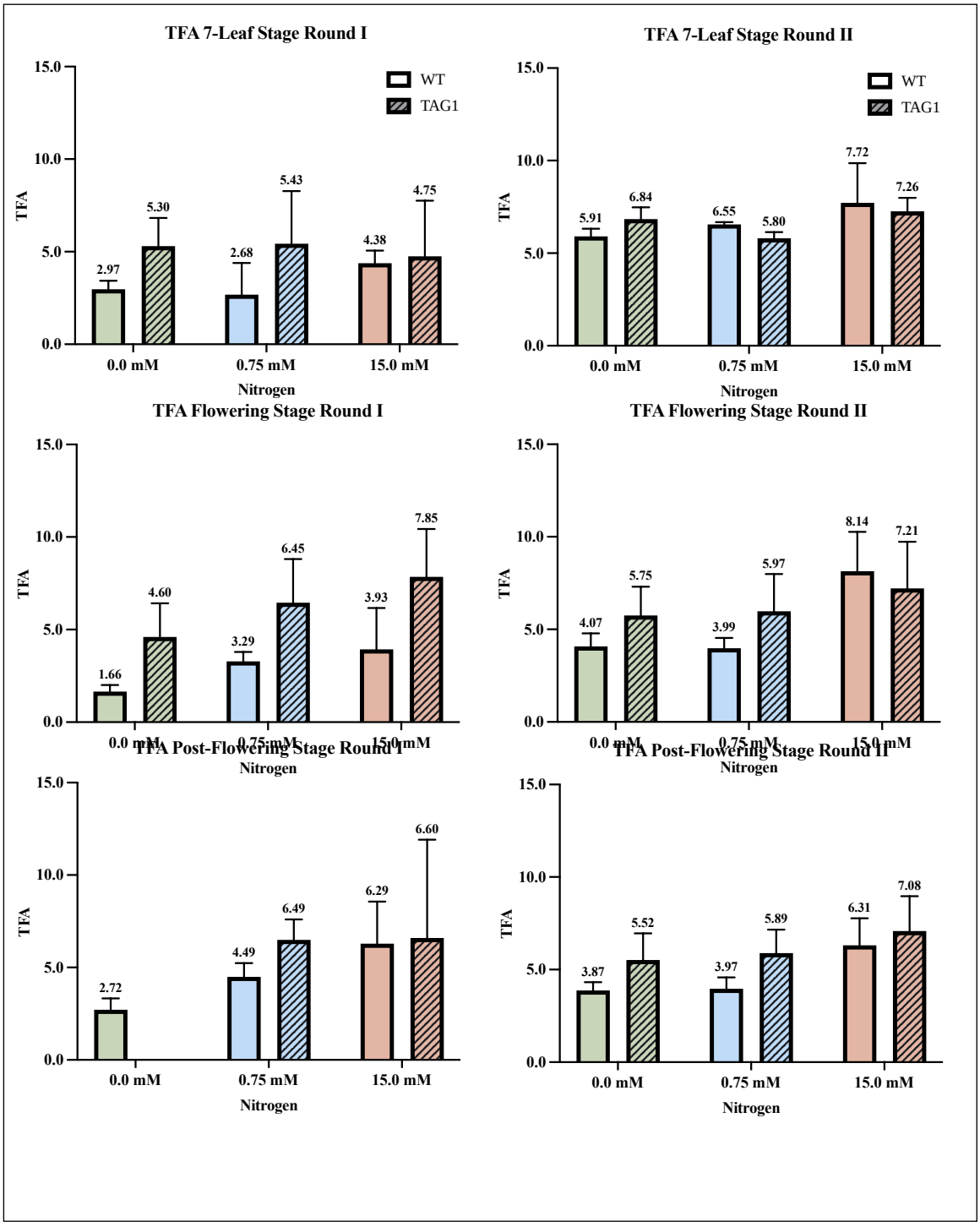

Figure 7. Interleaved bars graph illustrating total fatty acids (TFA) values in percentage per dry weight (%DW) across three different nitrogen levels, 0.0, 0.75 and 15.0 mM KNO_3. TFA content averages for two genotypes of *Sorghum bicolor*, WT and TAG1 are displayed for 2 rounds of the experiments separated and 3 developmental stages: 7-leaf, flowering and post-flowering (around 2 weeks after flowering). Error bars indicate standard deviations of the means.

Biomass

According to Table 3, in the 7-leaf growth stage, N availability had a significant effect on both the fresh weight (FW, $P<0.01$) and dry weight (DW, $P<0.01$) of the plants. The FW average values were 13.7, 20.4 and 30.3 g for 0.0, 0.75 and 15.0 mM KNO3, respectively. While the DW averages resulted in 2.0, 2.5 and 3.5 g for 0.0, 0.75 and 15.0 mM KNO_3. Although, the difference in averages for both FW and DW were only significant between treatments 15.0 and 0, 0 and 75 but not between 0.0 and 0.75. Additionally, a significant genotype effect is observed in FW ($P=0.02$) and DW ($P=0.04$). Block alone did not have a significant effect, and there was no significant interaction observed between nitrogen treatments and genotype. The dry weight percentage (DW%) values were not significantly different among the treatments.

In the post-flowering stage, both fresh and dry weight significantly increased with the increase of N availability ($P<0.01$). FW means for 0.0, 0.75, and 15.0 mM N treatments were, respectively, 28.3, 67.0 and 236.6 g. While for DW, the means were 10.2, 21.1 and 60.3 g, respectively. Dry weight percentage decreased with increasing in N availability. In summary, results show that the aboveground biomass of the plant significantly differed among the treatments.

Figures 7-8 illustrate that differences in means for FW and DW across nitrogen treatment have the same pattern in both developmental stages. Noticeably, in the post-flowering stage, FW had an elevated standard deviation from the means for the 15.0 mM

N treatment, which presented measurements that resulted in values almost twice higher as the means. The genotype effect demonstrated in Table 3 can also be observed in the figures.

Table 3. Impact of nitrogen availability on all aboveground biomass in *Sorghum bicolor* at the 7-leaf and post-flowering developmental stages for both rounds of experiments combined.

Aboveground Biomass	Nitrogen Treatment, mM[1]			*P*-value[2]				
	0.0	0.75	15.0	R	B	N	G	N × G
				7-Leaf Stage				
Fresh Weight, g	13.7[a]	20.4[a]	30.3[b]	-	0.32	**<.01**	**0.02**	0.41
Dry Weight, g	2.0[a]	2.5[a]	3.5[b]	-	0.62	**<.01**	**0.04**	0.85
DW, %	14.5[a]	12.6[a]	11.6[a]	-	0.51	0.09	0.99	0.87
				Post Flowering Stage				
Fresh Weight, g	28.3[a]	67.0[a]	236.6[b]	**<.01**	0.78	**<.01**	0.52	0.94
Dry Weight, g	10.2[a]	21.1[b]	60.3[c]	**<.01**	0.99	**<.01**	**0.03**	0.35
DW, %	36.1[a]	35.6[a]	32.2[b]	**<.01**	0.06	**<.01**	0.07	0.72

[1]0.0, 0.75 and 15.0 Mm KNO_3. [2]R = round effect; B = block effect; N = nitrogen effect; G = genotype effect; N × G = nitrogen × genotype interaction effect. Numbers followed by different letters represent significant differences at the $P \leq 0.05$. Means for 7-leaf stage are the results of 18 biological replicates; and means for post-flowering stage are the results of 90 biological replicates from both experiments rounds combined.

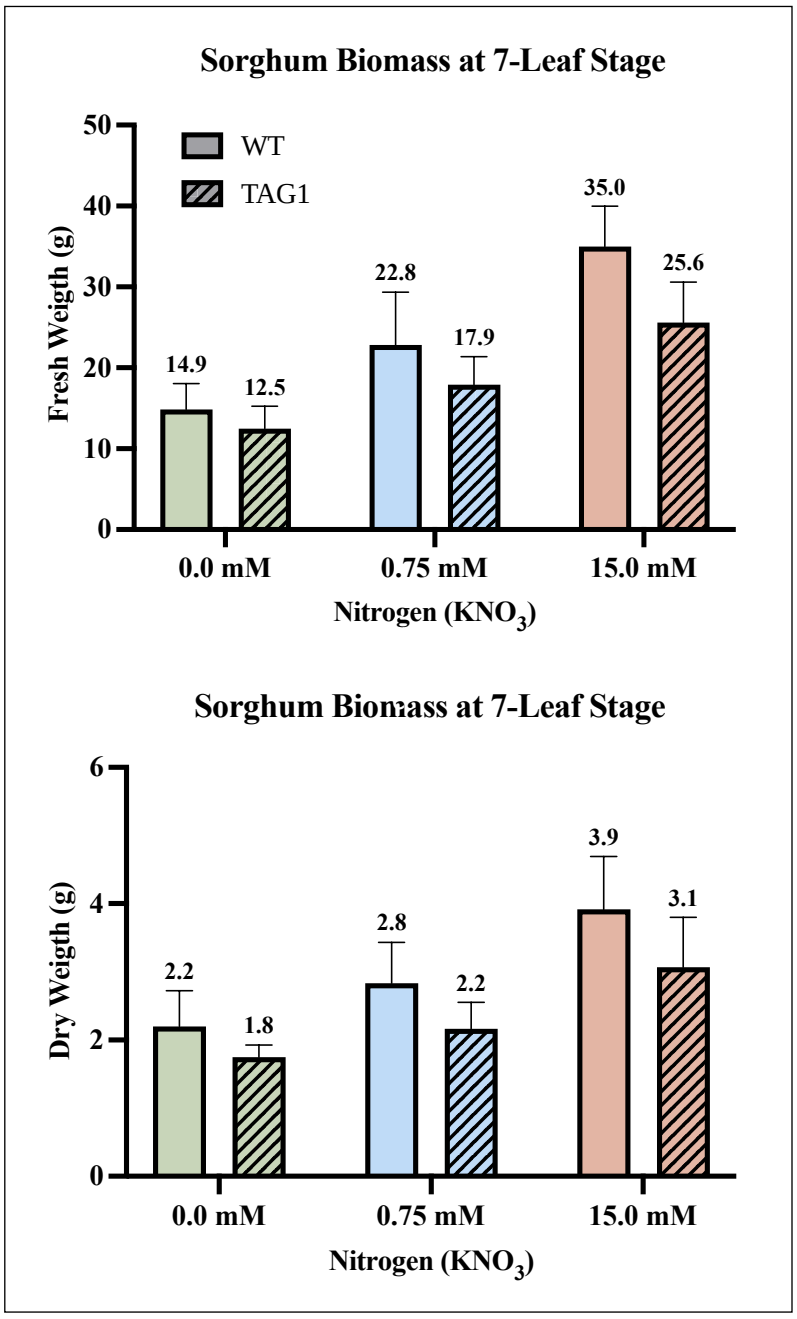

Figure 8. Interleaved bars graph illustrating fresh weight and dry weight means in grams across three different nitrogen levels, 0.0, 0.75 and 15.0 mM KNO_3. Biomass averages for two genotypes of Sorghum bicolor, WT and TAG1 are displayed for the 7-leaf developmental stage. Error bars indicate standard deviations of the means.

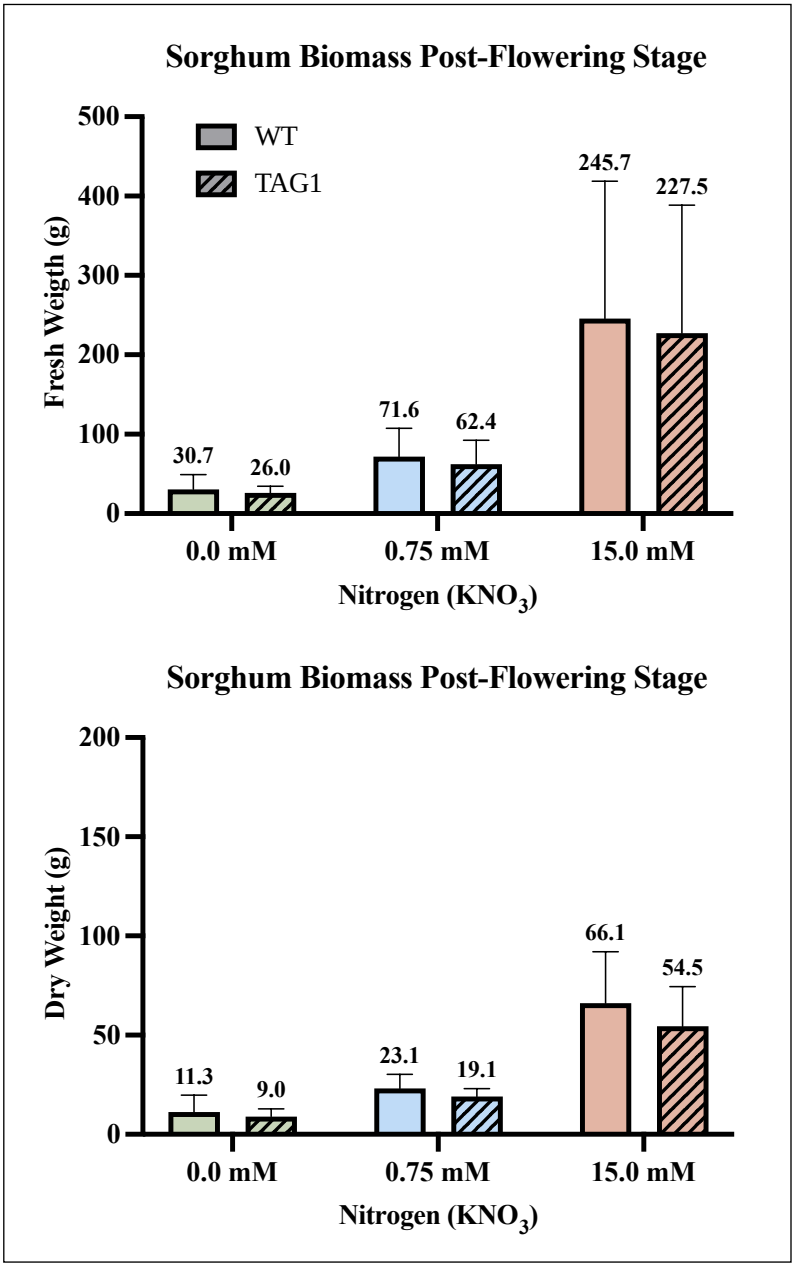

Figure 9. Interleaved bars graph illustrating fresh weight and dry weight means in grams across three different nitrogen levels, 0.0, 0.75 and 15.0 mM KNO_3. Biomass averages for two genotypes of Sorghum bicolor, WT and TAG1 are displayed for the post-flowering developmental stage (around two weeks after flowering). Error bars indicate standard deviations of the means.

In Table 4 are displayed the results of all aboveground biomass for each growing round of the experiment separated at the post-flowering stage. N effect on FW and DW for each round is the same as the one observed in the combined rounds (P<0.01). Genotype effect differs in FW between Round I and II, respectively, P=0.25 and P=0.02. However, is noticeable that biomass values presented in Round I are much higher than in Round II, explaining the elevated standard deviation from the means demonstrated in Figure 7.

Table 4. Impact of nitrogen availability on all aboveground biomass in *Sorghum bicolor* at the 7-leaf and post-flowering developmental stage for Rounds I and II of experiments analyzed separately.

Aboveground Biomass	Nitrogen Treatment, mM[1]			*P*-value[2]			
	0.0	0.75	15.0	B	N	G	N × G
	Post-Flowering Stage Round I						
Fresh Weight, g	25.9[a]	98.4[b]	428.4[c]	**0.03**	**<.01**	0.25	0.72
Dry Weight, g	5.6[a]	22.3[b]	85.3[c]	**0.01**	**<.01**	**<.01**	**0.03**
DW, %	24.8[a]	23.1[a]	20.1[a]	0.22	0.08	0.86	0.23
	Post-Flowering Stage Round II						
Fresh Weight, g	30.0[a]	46.1[b]	108.7[c]	0.36	**<.01**	**0.02**	0.74
Dry Weight, g	13.2[a]	20.3[b]	43.6[c]	**<.01**	**<.01**	**<.01**	0.42
DW, %	43.6[ab]	44.0[a]	40.2[b]	**<.01**	**0.02**	**0.01**	0.14

[1]0.0, 0.75 and 15.0 Mm KNO_3. [2]B = block effect; N = nitrogen effect; G = genotype effect; N × G = nitrogen × genotype interaction effect. Numbers followed by different letters represent significant differences at the $P \leq 0.05$. Means for post-flowering stage Round I are the results of 36 biological replicates; and means for post-flowering stage Round II are the results of 54 biological replicates.

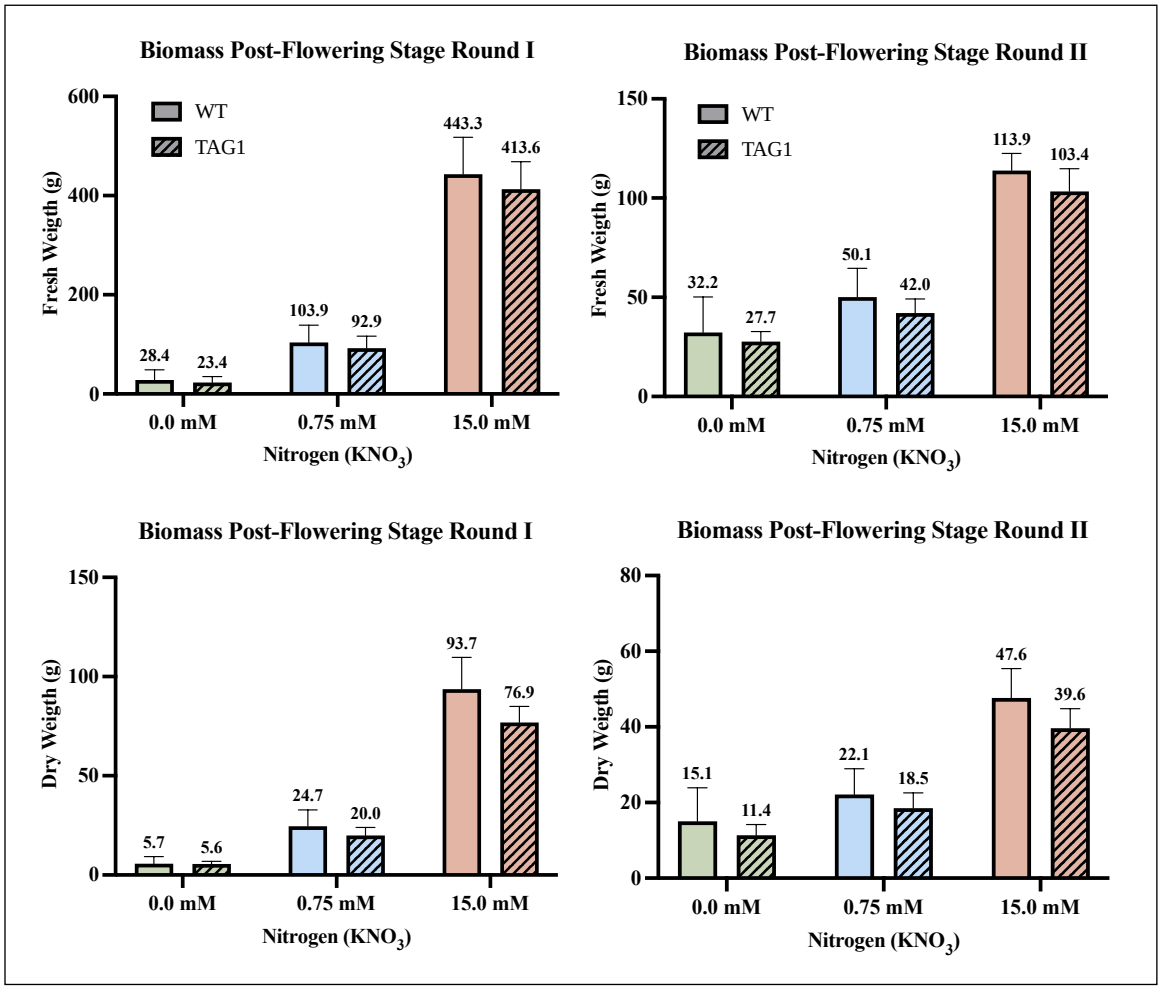

Figure 10. Interleaved bars graph illustrating fresh weight and dry weight means in grams across three different nitrogen levels, 0.0, 0.75 and 15.0 mM KNO_3. TAG content averages for two genotypes of *Sorghum bicolor*, WT and TAG1, are displayed for 2 rounds of the experiments separated and 3 developmental stages: 7-leaf, flowering and post-flowering (around 2 weeks after flowering). Error bars indicate standard deviations of the means.

Table 5 displays the results for vegetative aboveground biomass, excluding grains. For the combined analysis of both rounds, it was observed that all the statistical significance was the same as previously observed in the analysis including grains, with the exception of DW, where genotype did not have a significant effect (P=0.45).

Table 6 shows that when analyzing individual rounds, there are some differences in significance for block and genotype effects. But, most importantly, N treatment remains a significant effect on FW and DW (P<0.01). All the phenotypic differences leading to biomass variations observed in the study can be visualized in Figures 10-12.

Table 5. Impact of nitrogen availability on vegetative aboveground biomass (no grains) in *Sorghum bicolor* in the post-flowering developmental stage for both rounds of experiments combined.

Aboveground Biomass	Nitrogen Treatment, mM[1]			*P*-value[2]				
	0.0	0.75	15.0	R	B	N	G	N × G
	Post Flowering Stage							
Fresh Weight, g	19.7[a]	37.5[a]	128.3[b]	**<.01**	0.96	**<.01**	0.53	0.94
Dry Weight, g	6.6[a]	11.5[a]	34.4[b]	**<.01**	0.88	**<.01**	0.45	0.95
DW, %	33.3[a]	31.2[a]	27.3[b]	**<.01**	0.06	**<.01**	0.49	0.87

[1]0.0, 0.75 and 15.0 Mm KNO_3. [2]R = round effect; B = block effect; N = nitrogen effect; G = genotype effect; N × G = nitrogen × genotype interaction effect. Numbers followed by different letters represent significant differences at the $P \leq 0.05$. Means are the results of 90 biological replicates from both experiments rounds combined.

Table 6. Impact of nitrogen availability on vegetative aboveground biomass (no grains) in *Sorghum bicolor* in the post-flowering developmental stage for Rounds I and II of experiments analyzed separately.

Aboveground Biomass	Nitrogen Treatment, mM[1]			*P*-value[2]			
	0.0	0.75	15.0	B	N	G	N × G
	Post-Flowering Stage Round I						
Fresh Weight, g	15.3[a]	44.5[b]	202.2[c]	0.19	**<.01**	0.46	0.81
Dry Weight, g	4.7[a]	12.4[b]	52.9[c]	0.09	**<.01**	0.90	0.65
DW, %	30.9[a]	27.5[ab]	26.3[b]	0.09	**0.02**	0.30	0.13
	Post-Flowering Stage Round II						
Fresh Weight, g	22.6[a]	32.9[b]	79.0[c]	0.46	**<.01**	**0.05**	0.68
Dry Weight, g	7.9[a]	11.0[a]	22.2[b]	**<.01**	**<.01**	**0.04**	0.59
DW, %	34.8[a]	33.5[a]	27.9[b]	**<.01**	**<.01**	0.07	0.17

[1]0.0, 0.75 and 15.0 Mm KNO_3. [2]B = block effect; N = nitrogen effect; G = genotype effect; N × G = nitrogen × genotype interaction effect. Numbers followed by different letters represent significant differences at the $P \leq 0.05$. Means for Round I are the results of 36 biological replicates and means for Round II are the results of 54 biological replicates.

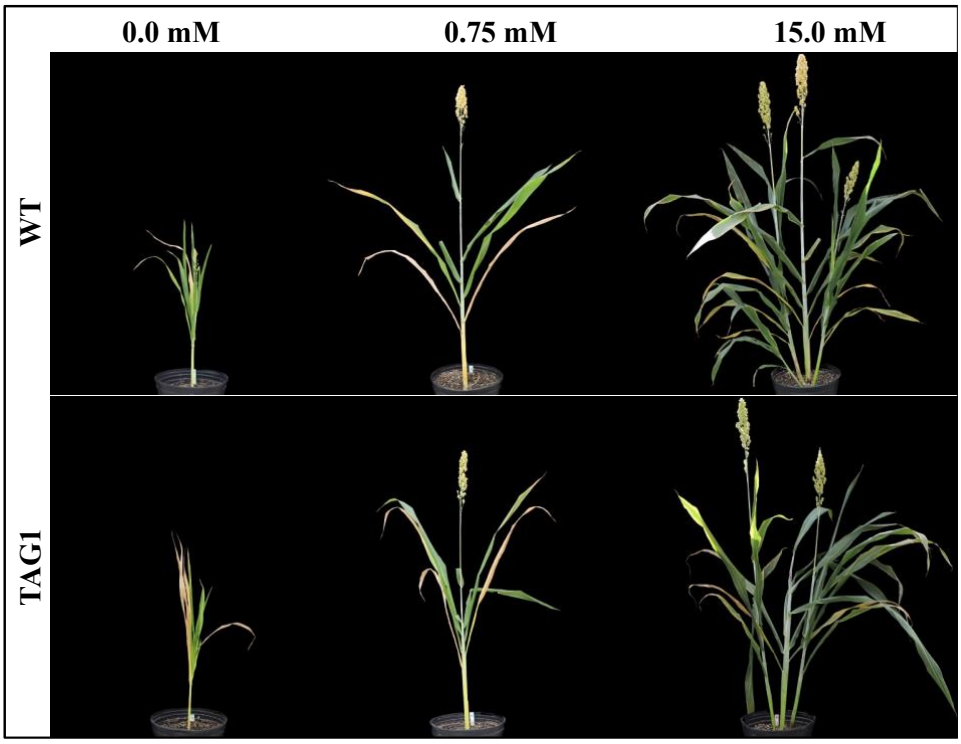

Figure 11 - Comparison of average phenotype for each genotype, WT and TAG1, at the three nitrogen treatment levels, captured at the post-flowering stage.

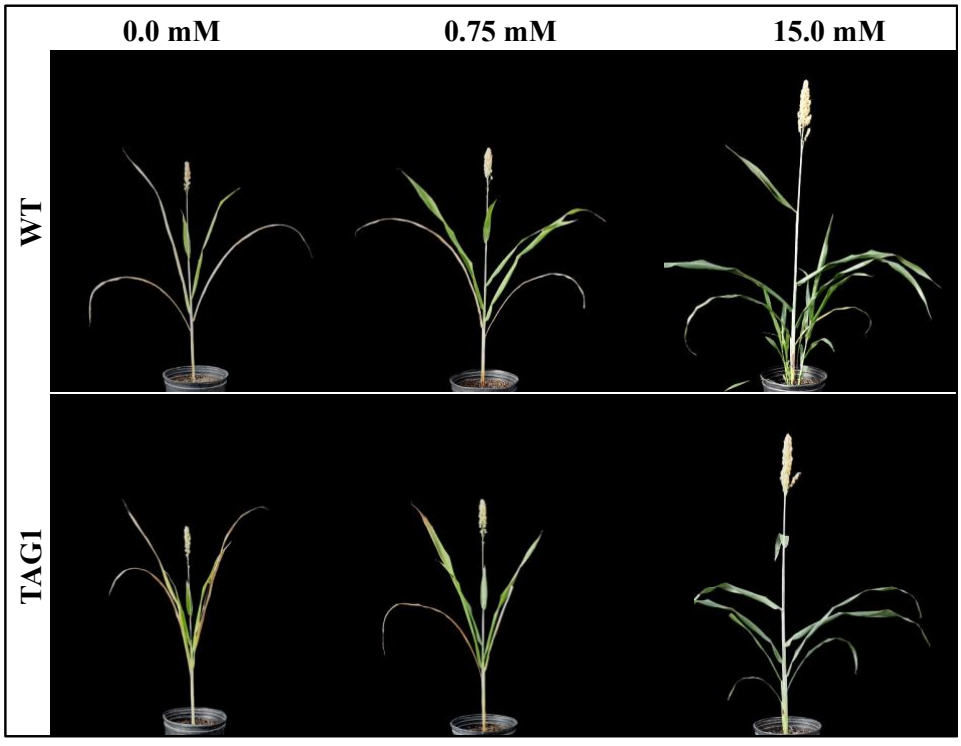

Figure 12 - Comparison of average phenotype for each genotype, WT and TAG1, at the three nitrogen treatment levels, captured at the post-flowering stage.

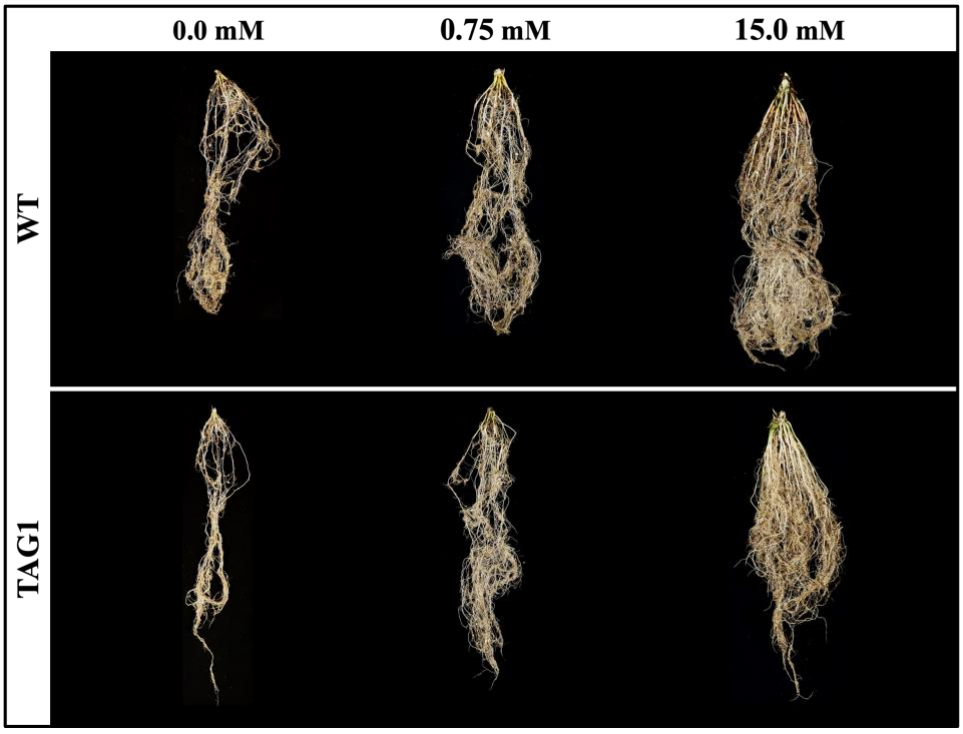

Figure 13. Comparison of average roots phenotype for each genotype, WT and TAG1, at the three nitrogen treatment levels, captured at the post-flowering stage.

Lipid Droplets

Although an attempt was made to capture lipid droplets in the vegetative tissues, the confocal imaging did not yield the expected results, as shown in Figures 13-14. The visualization of lipid droplets (red channel) does not follow a consistent pattern across different nitrogen levels and genotypes. The presence of droplets is not correlated to the TAG or TFA vegetative accumulation observed in this study. The significant difference in TAG levels between wild type and TAG1 lines (Table 1, Figure 3) is not noticeable in the captured images.

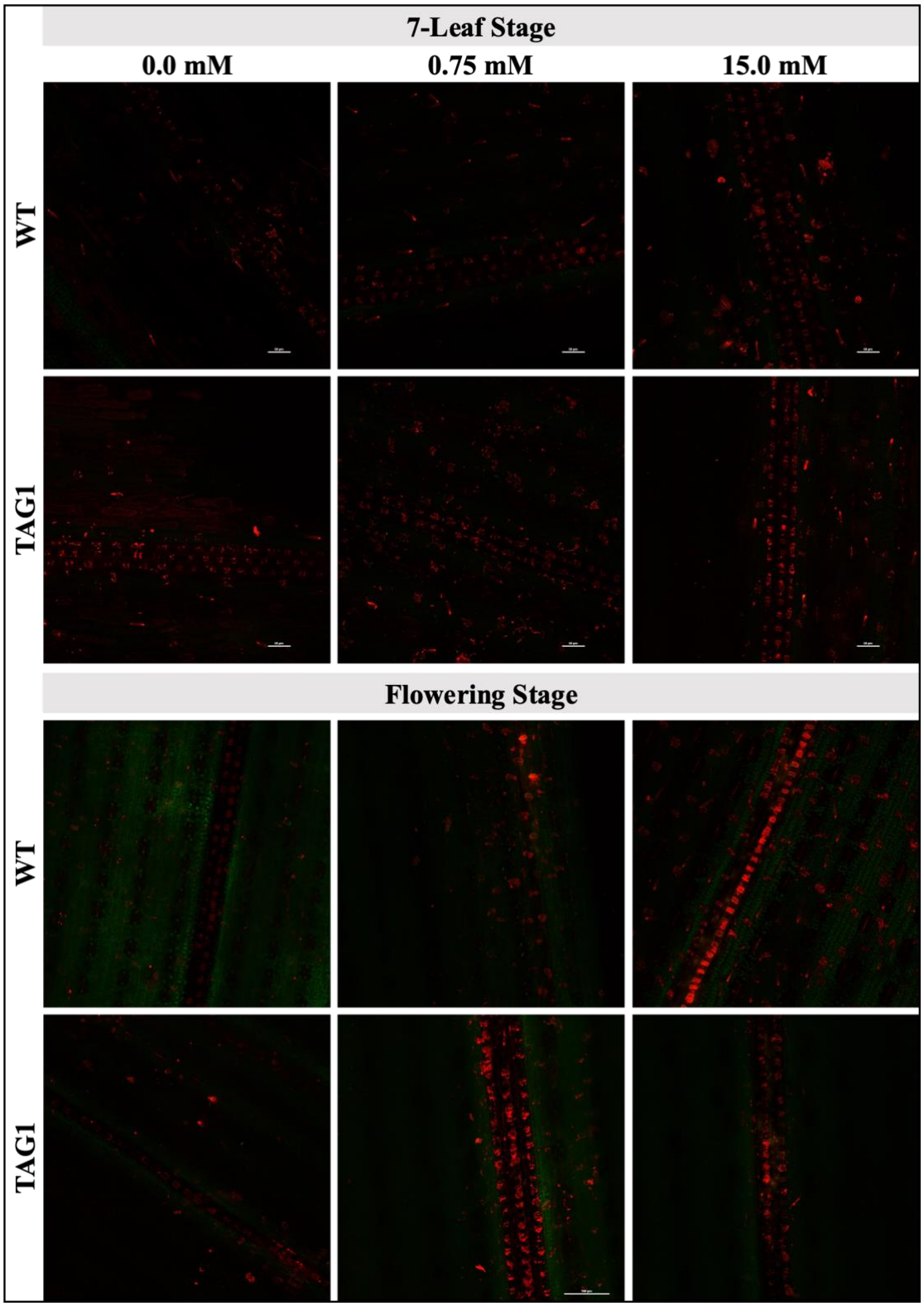

Figure 14. Confocal images showing the accumulation of lipid droplets in *Sorghum bicolor* fresh leaf sections at 7-leaf and flowering stages for wild-type and TAG1 lines. Lipid droplets were stained with Nile Red (red channel), while autofluorescence was used to highlight the chloroplasts (green). Scale bars correspond to 50 μm (7-leaf stage) and 100 μm (flowering stage).

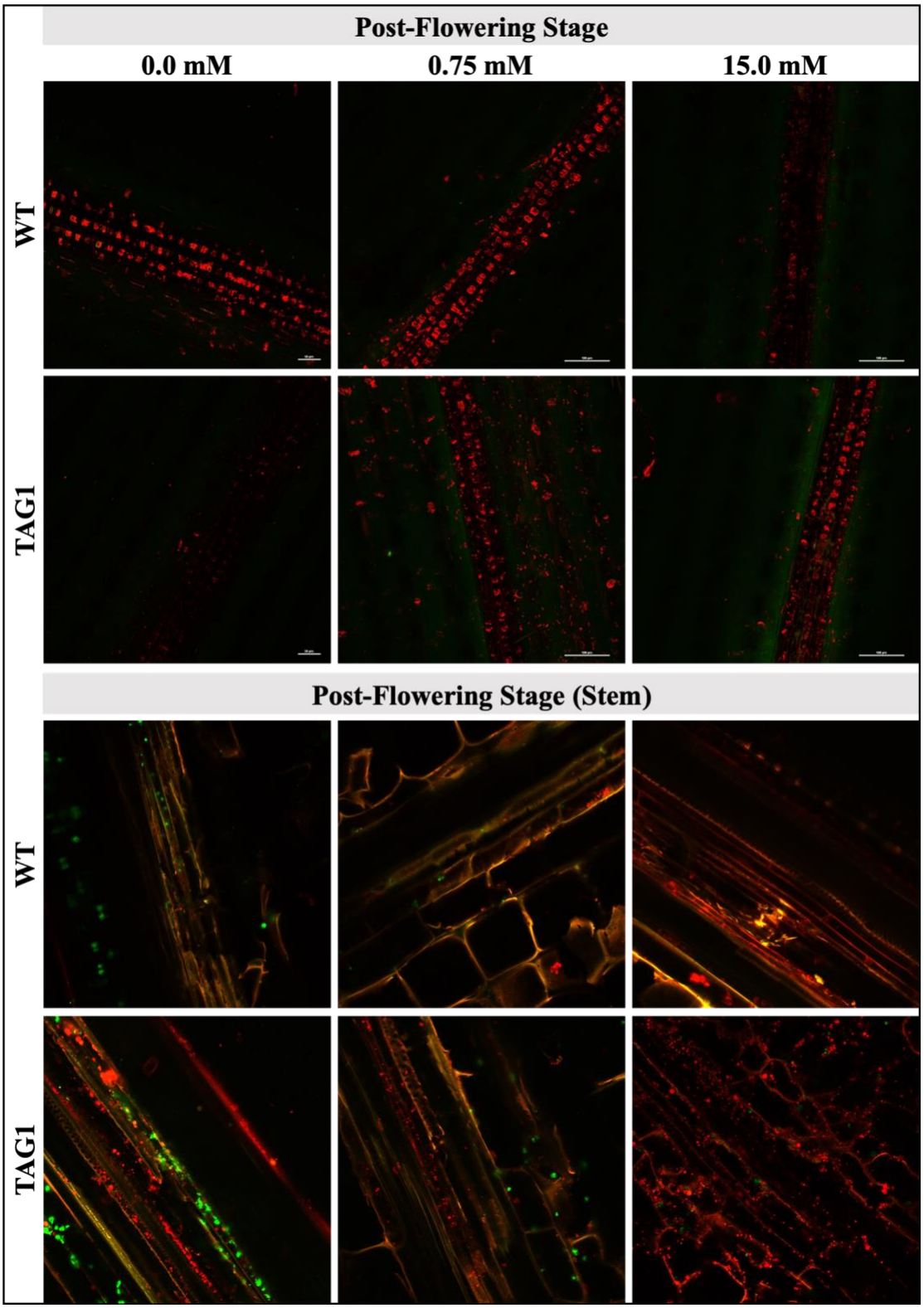

Figure 15. Confocal images showing the accumulation of lipid droplets in *Sorghum bicolor* fresh leaf and stem sections at the post-flowering stage for wild-type and TAG1 lines. Lipid droplets were stained with Nile Red (red channel), while autofluorescence was used to highlight the chloroplasts (green). Scale bars correspond to 50 μm and 100 μm.

Leaf Nitrogen, Chlorophyll and Photosynthesis

The variables presented in Table 7 were measured to exclude those parameters as the cause of vegetable lipids variations within the combination of treatment and genotype. As expected, nitrogen availability had a high impact on chlorophyll content ($P<0.01$), while photosynthetic rates did not differ between treatments 0.75 and 15 mM N. No data were obtained for 0.0 mM N treatment due to the size of leaves presented on those replicates. Leaf nitrogen content percentage was positively correlated with the offer of nitrogen. However, it did not have a considerable standard deviation from the obtained means, as observed in the vegetative lipids previously (Figure 15).

Table 7. Impact of nitrogen treatment leaf nitrogen content, chlorophyll content and photosynthesis rates in *Sorghum bicolor* in the post-flowering developmental stage for both rounds of experiments combined.

Variable	Nitrogen Treatment, mM[1]			*P*-value[2]			
	0.0	0.75	15.0	B	N	G	N x G
Leaf Nitrogen, %	0.98[a]	1.29[b]	2.69[c]	0.11	**<.01**	**0.04**	0.14
Chlorophyll, SPAD	26.3[a]	41.5[b]	57.5[c]	**0.03**	**<.01**	0.59	0.35
Photosynthetic Rate							

[1]0.0, 0.75 and 15.0 Mm KNO_3. [2]B = block effect; N = nitrogen effect; G = genotype effect; N × G = nitrogen × genotype interaction effect. Numbers followed by different letters represent significant differences at the $P \leq 0.05$.

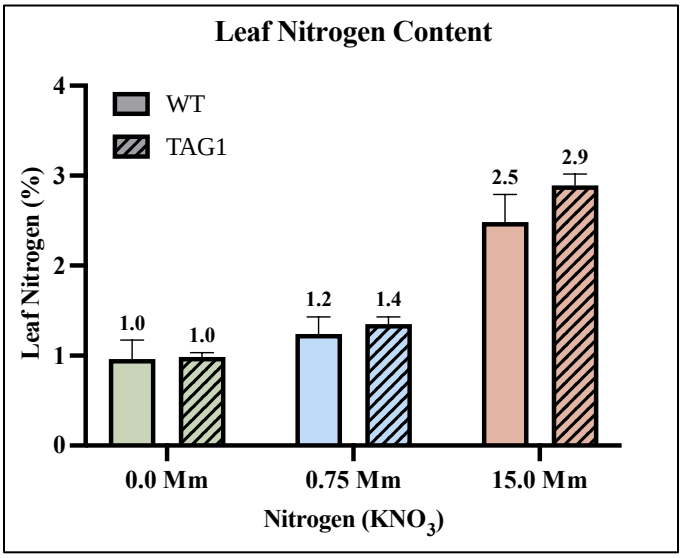

Figure 16. Interleaved bars graph illustrating nitrogen leaf content in percentage across three different nitrogen levels, 0.0, 0.75 and 15.0 mM KNO_3.

DISCUSSION

Despite the best efforts to control the environment in a greenhouse experiment, the time of year for planting certainly affected the response of the variables measured, biomass and lipids. By analyzing the means for biomass and the plant phenotypes captured during the two growing rounds of this study, it's possible to observe that Round II had etiolated plants resulting in less biomass yield, presenting about half the TAG content compared to Round I. The phenotypic difference can be observed in Figures 10-12 and Tables 3-6, where the means for all aboveground biomass FW for N treatments 0.0, 0.75 and 15.0 mM were, respectively: 25.9, 98.4 and 428.4 g on Round I and 30.0, 46.1, 108.7 g on Round II. This difference in biomass between growing rounds can also be a result of nutrient intake variances due to irrigation being offered as needed. Although the same volume of water was offered to all plants, Round II was cultivated at a time of the year with more cloudy days. Thus, lower irrigation frequency was needed when compared to Round I, which also reduces the availability of N and nutrients present in the Hoagland's solution to the plant.

The response of TAG to the limitation of nitrogen availability found in the studies correlates to what has been reported in previous microalgae studies (Converti et al., 2009; Chen et al., 2011; Taleb et al., 2017; Shaikh et al., 2019; Andeden et al., 2021; Arguelles & Martinez-Goss, 2021). In the 7-leaf and flowering stages, a decrease in TAG content was observed with the increased availability of N for the combined and individual analyses of experiment rounds. When discussing the post-flowering stage, the combined analysis is not the best option for analyzing the results since Round I did not yield results for the 0.0

mM N treatment for TAG1 line. But it is noticeable that for the WT in Round I, the tendency in TAG response to N was the same as the one observed in Round II post-flowering and previous developmental stages. Despite the lower number of replicates and small scale of this study, initial findings demonstrate that N limitation has an impact on TAG accumulation sorghum, suggesting that the same results previously seen in microalgae could possibly apply to higher plants (Table 1-2).

Vegetative lipids values are presented as means of three replicates for each treatment × genotype combination. However, is evident from Figures 3-6 that the standard deviation of means is significant for both WT and TAG1. This high variance has an impact on the non-significance observed between treatments, despite a numerical indication of the influence of N availability on lipids. Since other measured variables did not positively correlate to this variance (Table 7), increasing the number of replicates would contribute to enhancing the accuracy of the results and reduce data variance per treatment. Furthermore, conducting this study in a field setting, where non-controlled variables are present, would provide a more realistic depiction of the results.

As expected, nitrogen had a crucial impact on FW and DW for biomass yields. Nitrogen availability rates considered in this experiment were extreme scenarios aimed at building a curve for lipids response to the limitation of the nutrient. A more realistic agronomic nitrogen application scenario could be investigated to determine the point where the trade-off between lower nitrogen application and biomass yields is non-longer

advantageous. As well as economic viability studies to balance fertilization costs, oil production per biomass and total biomass production.

When observing the capture of lipid droplets through confocal microscopy, inconsistent patterns may be due to the angle at which the leaf or stem tissue was cut and mounted in the slides. Vanhercke et al. (2018) conducted a study that also included imaging lipid droplets, and the methodology and figures presented in the investigation suggest that a cross-section could have provided a clearer visualization of the mesophyll and lipid droplets. It is worth noting that the images presented in the study are derived from one of the three biological replicates that were used for imaging. Although the selected image aimed to represent the average of the three captured images, it is crucial to acknowledge that there were also visual differences between the images, and only one was chosen for representation.

CONCLUSIONS

The findings of this study suggest that strategical management of nitrogen in sorghum plants holds promising potential for the production of a sustainable feedstock crop for biofuel. Although not always statistically significant, nitrogen starvation has been numerically found to impact triacylglycerol content in higher plants, as observed previously in microalgae studies. A curve representing the response of TAG to extremes availability of N, such as 0.0. 0.75 and 15.0 mM of KNO_3 in solution provides insights for future studies and potential efficient fertilization strategies.

Nitrogen plays a crucial role in biomass yield, therefore, further investigations into the delicate balance between limited nitrogen application and biomass production could lead to the development of a sustainable management strategy for sorghum cultivation. This strategy would aim to reduce nitrogen usage while maintaining or increasing oil production. Such an approach would not only improve the efficiency of biofuel production but also contribute to the overall sustainability of the agricultural industry.

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