**Running title:** Canopy temperature of high N cotton

# Canopy temperature of high-nitrogen water-stressed cotton

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**Abbreviations:**

ABA, abscisic acid; ABA-GE, abscisic acid glucose ester; ACRI, Australian Cotton Research Institute; DAS, days after sowing; HVI, High Volume Instrument; GAM, generalised additive model; NUE, nitrogen use efficiency; REML, residual maximum likelihood; Tc, canopy temperature; VPD, vapour pressure deficit; WUE, water use efficiency; $\psi_{leaf}$, leaf water potential.

## ABSTRACT

Australian cotton (*Gossypium hirsutum* L.) farmers are adopting canopy temperature (Tc) based irrigation scheduling as a decision support tool to improve on-farm production. High nitrogen supply, characteristic of the high-yielding, furrow irrigated cotton system of Australia, might alter cotton Tc with implications for irrigation. We examined growth, physiological and biochemical traits and changes in Tc of well-watered and water-stressed cotton plants supplied high to excessive levels of nitrogen under glasshouse conditions. We also examined Tc, lint yield and fibre quality of furrow irrigated cotton crop supplied with high nitrogen. In the glasshouse and under well-watered conditions, high nitrogen supply stimulated plant growth, increased stomatal conductance and photosynthesis resulting in cooler Tc. Under water deficit stress high nitrogen also stimulated growth, increasing plant water demand and thus vulnerability to water stress, which manifested as warmer Tc. Water stressed plants supplied high nitrogen also showed reduced stomatal conductance, lower leaf water potential, and greater accumulation of leaf and xylem sap abscisic acid. Furrow irrigated crops supplied higher nitrogen also had higher Tc, but there was no gain in lint yield and fibre quality. The influence of high nitrogen on cotton Tc suggests the need for accurate and reliable Tc-based irrigation scheduling is paramount.


## INTRODUCTION

Irrigated cotton production in Australia is intensive, broadacre cropping, characterised by high nitrogen fertiliser application. Despite the recommendation of 200 kg N $ha^{-1}$ to achieve optimal crop nitrogen use efficiency (NUE) (Rochester, 2011), almost 50% of farmers apply more than 50 kg N $ha^{-1}$ above the recommendation (Roth, 2013). Roth (2013) in a 2013

survey of irrigated cotton farms reported that nitrogen rates varied between 93 to 370 kg N $ha^{-1}$ with 46% of farmers applying 250 kg N $ha^{-1}$ or more. The main reason for the high nitrogen application is as insurance against nitrogen-deficit related yield loss (Roth, 2013). This over application of nitrogen unnecessarily increases the cost of production, nitrogen leaching (Macdonald et al., 2016c), runoff losses (Silburn and Hunter, 2009; Macdonald et al., 2017), and the potential for greenhouse gas emissions (Macdonald et al., 2016a, b), as well as reducing NUE (Rochester, 2011). In contrast to high nitrogen application, water use in the Australian cotton production system is constrained by its scarcity and increasing competition from other sources (Richards et al., 2008). This has compelled farmers to adopt more efficient use of water and concerted efforts to further improve water use efficiency (WUE). There are several projects the Australian cotton industry has invested in to optimise WUE and NUE in order to maximise their profits. For reviews of such projects see Roth et al. (2013) and Rochester (2011), respectively.

One method for improving WUE, which is gaining acceptance by the industry, is to adopt canopy temperature (Tc) based irrigation scheduling (Conaty et al., 2012, 2015). This approach is favoured because it is plant-based, equipment required is relatively cheap and easy to operate, it provides continuous data on plant water status and is suited to the long irrigation intervals (>5 days) characteristic of Australian furrow irrigated systems. Most (92%) cotton farms in Australia are furrow irrigated (Roth, 2015). Canopy temperature-based irrigation scheduling can also be easily incorporated with other currently used irrigation scheduling techniques, which are mainly soil-based (soil moisture capacitance probes and neutron soil moisture probes, used by 57 and 22% of farmers, respectively) (Roth 2011).

Under non-water limiting conditions, plants transpire water from open stomata, mostly on leaf surfaces. The loss of water and latent heat from plant leaves and the crop canopy cools the leaves and canopy. In contrast, under water deficit conditions, plants minimise water loss from leaves by inducing gradual stomatal closure and reduction in stomatal conductance to water, which in turn limits evaporative cooling. This limitation causes a rise in leaf and canopy temperature. Researchers have exploited this knowledge to develop sensors that monitor Tc and protocols for optimising irrigation scheduling based on Tc. The initial application of Tc for irrigation scheduling used Tc derived indices including stress degree day (Idso et al., 1977; Jackson et al., 1977), crop water stress index (Idso et al., 1981; Jackson et al., 1981), temperature stress day (Gardner et al., 1981), and canopy temperature variability (Clawson and Blad, 1982). Later the stress time temperature threshold approach was developed for irrigating cotton in parts of the USA (Wanjura et al., 1995; Upchurch et al., 1996; Wanjura and Upchurch, 1997; Wanjura et al., 2004). This approach was based on comparing Tc against a pre-determined threshold temperature and triggering irrigation when Tc exceeds the threshold for a specified period of time provided atmospheric conditions will allow for transpirational cooling to occur, i.e. cumulative Tc stress duration (Mahan et al., 2005). The threshold Tc for cotton was taken as 28 to 29°C, the optimum for cotton enzymatic and physiological function (Mahan et al., 2005; O'Shaughnessy and Evett, 2010; Conaty et al., 2012). In some other research fields, the principles underlining changes in Tc have been similarly applied i.e. Tc of the subject of interest is compared against a predetermined reference to assess crop health and maturity. This form of application of Tc data might be inappropriate if there are other factors that influence Tc independently, such as growth environment temperature, vapour pressure deficit (VPD), and nitrogen status of the plant. Whilst the latter is amenable to manipulation for positive outcomes, some others are

not. Radin and Ackerson (1981) showed that nitrogen deficiency reduced stomatal conductance by inducing stomatal closure. Inhibition of stomatal conductance results in warmer Tc.

Abscisic acid (ABA) is a multifunctional plant hormone readily occurring in vascular tissue, as well as parenchyma cells outside vascular bundles. It plays roles in germination, seasonal growth patterns, and importantly in stress responses – including water deficit, salinity, cold temperatures and frost (Vishwakarma et al., 2017). ABA production results in responses that help protect plants from these stressors. During prolonged periods of drought stress, catabolism of ABA occurs continuously, but is balanced by de novo biosynthesis to maintain high ABA levels until the stress is alleviated (Harrison and Walton, 1975; Ren et al., 2007). The phytohormones ABA is catabolised through either conjugation to produce ABA-glucose ester (ABA-GE) or oxidation to form phaseic acid, which is further metabolised to inactive dihydrophaesic acid (Sharkey and Raschke, 1980; Zeevaart, 1980). Phaseic acid can trigger similar plant responses to ABA, including stomatal closure; however, its bioactivity in terms of stomatal behaviour is much weaker than ABA and unlike ABA the phaseic acid response can vary significantly across species (Sharkey and Raschke, 1980). Stomatal conductance is influenced by a range of factors including water and nutrient status, and it is linked to concentration of ABA (Chaves et al., 2002; Pantin et al., 2012). For example, water stress increases endogenous ABA concentration, which acts directly on the guard cells, causing stomatal closure (Sharkey and Raschke, 1980; Zeevaart and Creelman, 1988). Also, nitrate (the predominant form of soil available nitrogen to plants) deficiency increases ABA concentration resulting in decreased stomatal conductance (Radin et al., 1982; Wilkinson et al., 2007).

In this study it was hypothesised that high nitrogen availability will affect cotton canopy temperature, particularly where plants are exposed to water deficit through the wetting and drying cycles characteristic of furrow irrigation. Thus, the aim of our study was twofold: 1) To determine if high to excessive nitrogen supply influences cotton Tc under water stress; and 2) To develop an understanding of how water and nitrogen availability alters leaf-level physiology, plant hormone and biochemical status and plant growth parameters associated with changes in Tc. To achieve this, we investigated changes in plant growth, leaf gas exchange, leaf and xylem sap ABA and leaf water potential, as well as Tc of cotton grown under different nitrogen and water levels in complimentary glasshouse and field experiments. This study is important as current Tc based irrigation scheduling protocols may need to be adapted to mitigate the possible associations between high nitrogen conditions, plant water status and Tc.

## MATERIALS AND METHODS

### Location and Experiments

From 2015 through 2016 a glasshouse experiment, Experiment I, and a field experiment, Experiment II, were conducted at the Australian Cotton Research Institute (ACRI). The ACRI station (30°12′ S, 149°36′E) is 22 km north-west of Narrabri, NSW, Australia. Experiment I was sown during the off-season in June 2015, while Experiment II was sown in October 2015 for the 2015/2016 cotton season to confirm the glasshouse results.

### Glasshouse experimental design and crop husbandry

About 25 seeds of cotton (*Gossypium hirsutum* L. cultivar Sicot 71BRF) were sown into 8 L plastic pots (0.25 m in diameter) filled with soil. The soil was a mixture of 1.0 kg of sand and

7.5 kg of grey clay (vertosol, Australian soil taxonomy; Typic Haplustert, USDA Soil Taxonomy; [Isbell 1996]), obtained from cotton fields at ACRI. The soil at ACRI is generally 60 to 65% clay fraction, of low drainage rate (Weaver et al., 2005), pH range of 8.0 to 8.8, and low in organic matter and nitrogen (Milroy and Bange 2013). To improve the nutrient status of the soil 10 g of MULTIgro® (Incitec Pivot Fertilizers, Melbourne, Australia) basal fertiliser was mixed with the soil before filling into pots. MULTIgro is a multi-purpose garden fertiliser with nutrients N, P, K, S, and Ca of 13.1, 4.5, 7.2, 15.4, and 2.4%, respectively. Soils in pots were kept saturated with water for 48 h prior to sowing. After germination seedlings at the three-leaf stage were thinned to seven per pot.

Plants were grown in a glasshouse with temperature set to 18°C at night (1900 – 0500 h) and 35°C during the day by an automated heating and cooling system. Air temperature and relative humidity at plant height were monitored with tinytag data loggers (model TGU 4017 Gemini Data Logger Ltd, West Sussex, UK). Radiation data was obtained from a weather station at ACRI. Plants were kept well-watered by automatically applying ~80 ml of tap-water to saucers underneath pots at 0900 h every day. This was initially programmed for once a day at 0900 h then as the plants grew another application was added at 1600 h. Plants were sprayed with commercial pesticide (Agrimec®, Syngenta UK Ltd, Cambridge, UK) prior to flowering to control Silverleaf whiteflies (*Bemisia tabaci* Gennadius) and aphids (*Aphis gossypii* Glover.), following standard glasshouse practice at ACRI.

The experimental design was a randomised complete block with three replicates. It was laid out as a 2 by 3 factorial design with two water levels (well-watered and water deficit stressed) and three nitrogen levels (basal, basal + 200 and basal + 300 kg N $ha^{-1}$). Basal being the 10 g of MULTIgro® while the extra nitrogen was supplied as urea. Potted plants were

laid out across three glasshouse benches, with each bench being a replicate containing all six treatment combinations. Each treatment on a bench was represented by a set of four pots of plants, this is our experimental unit. The pots were kept together to achieve a closed canopy. Each pot had seven plants at the beginning of the experiment. Destructive sampling for biomass at different times reduced the plant population and prevented overcrowding. The extra nitrogen was applied as a single application at 60 DAS. The three nitrogen levels i.e. basal, basal + 200 and basal + 300, will henceforth be referred to as control-N, 200 N and 300 N, respectively.

The water-deficit stress treatment involving two stress cycles was initiated at flowering (65 DAS) by withholding water from plants for seven consecutive days. After the first 7-day stress, plants were kept well-watered for the next seven days to encourage recovery from stress before reinitiating a second 7-day period of water stress.

**Soil moisture content**

Soil moisture content was determined with a soil moisture sensor (ThetaProbe Type ML2x, Delta-T Devices Ltd, Cambridge, UK). The sensor comprised of four rods, with three rods arranged in a circle around a central rod. Each rod was 3mm in diameter, made of stainless steel, and with sharpened tips. Accuracy of the sensor was ±0.05 $m^3$ $m^{-3}$ (5%) for 0-70°C and ±0.01 $m^3$ $m^{-3}$ (1%) for 0-40°C. Measurements were taken on days 6 and 7 during a stress regime, between 0800 and 1000 h, by inserting the whole length of the sensor's rods into soils in pots used to grow the plants. Soil moisture content was measured in four pots per treatment in each of the three blocks.

**Canopy temperature**

A total of 18 wireless, solar-powered, infra-red thermometers (ARDUCrop, CSIRO, Canberra, Australia) were used to continuously monitor Tc from first square (40 DAS) to flowering (86 DAS). The infra-red thermometer was mounted and maintained at 20–30 cm above the crop canopy (four to seven plants positioned closely together). Canopy temperature was recorded every minute. For detailed description of the ARDUCrop sensor construction, data acquisition and processing see Jones et al. (2017).

**Plant height, leaf area and above-ground biomass**

Four plants per experimental unit (one per pot) were harvested (cut off at ground level) at 43, 64, 70 and 86 DAS. Three of these plants, representative of the experimental unit, were used for determination of plant height, leaf area and above-ground biomass. The fourth plant was discarded due to limited processing resources and to avoid overcrowding. Height of the three harvested plants were measured. Harvested plants were separated into leaves, stems, squares and bolls. Leaf area was determined using a planimeter (model LI-3100 Area Meter; Li-Cor Inc. Lincoln, Nebraska, USA). Dry weight of harvested plants parts was determined after drying at 70°C until constant weight in a forced-fan oven.

**Leaf water potential**

One youngest fully expanded leaf (from the terminal bud of the main stem) per experimental unit was used to determine solar noon leaf water potential ($\psi_{leaf}$) according to Scholander et al. (1965). The leaf was cut at the base of the petiole and placed in a Scholander-type leaf pressure chamber (model 1515D, PMS Instrument Co. Albany, OR, USA), with the petiole exposed. The pressure inside the chamber was increased by supplying nitrogen gas until sap

became visible on the cut surface of the exposed petiole. The pressure at which sap became visible was recorded as the $\psi_{leaf}$. For the water deficit stressed plants determining $\psi_{leaf}$ was difficult and for a few of them the sap exuded was greenish (not colourless), suggesting a mixture of apoplastic fluid. These samples were not discarded but still used to determine $\psi_{leaf}$ and later for xylem sap ABA analysis (described below).

**Leaf-level physiology**

Leaf level stomatal conductance, photosynthesis and transpiration were measured on the youngest fully expanded leaf of 32 plants (1-3 measurements per experimental unit) on the 6th day of a water stress regime. Measurements were made within one hour of solar-noon with infra-red gas analysers (LI-6400XT, Li-Cor Biosciences, Nebraska, USA). Cuvette parameters were set at 2000 µmol $m^{-2}s^{-1}$ photosynthetically active radiation, 400 µmol $CO_2$ $mol^{-1}$ air for the reference air $CO_2$ concentration and flow rate of 500 µmol $s^{-1}$. Sample chamber relative humidity was maintained between 40 and 70%. The block temperature was set to 32°C to reflect the prevailing conditions of the glasshouse at the time of measurement.

**Leaf ABA, ABA-GE and phaseic acid**

Eighteen young unfurled leaves, one per block per treatment, were collected on day 7 of a water stress regime before pots were watered, to measure ABA, ABA-GE, and phaseic acid. Sampled leaves were snap frozen in liquid nitrogen then transferred to -80°C freezer for storage until analysis. ABA, ABA-GE, and phaseic acid were determined according to the methods described in Speirs et al. (2013). Briefly, 50 mg of frozen leaf tissue were ground under liquid nitrogen, extracted overnight in 20% aqueous methanol at 4°C then centrifuged. Deuterated ABA, ABA-GE and phaseic acid, were added to the supernatant. The compounds

of interest were isolated using Phenomenex SPE columns (60 mg/ml: 8B-S100-UAK); equilibrated as per the manufacturer's directions prior to sample loading, then washed with 20% aqueous methanol, and eluted with 90% aqueous methanol. An aliquot of the eluate was dried in a vacuum centrifuge in preparation for analysis.

Analysis of ABA, ABA-GE and phaseic acid was undertaken by liquid chromatography/mass spectrometry (LC/MS/MS). The dried extracts were dissolved in 50 µl aqueous acetonitrile (10% with 0.05% acetic acid) and 20 µl was analysed by LC-MS/MS (Agilent 6410 QQQ LC-MS/MS with Agilent 1200 series HPLC). Separations were carried out on a Phenomenex C18(2) at 40°C using a gradient elution with nanopure water and acetonitrile, both with 0.05% acetic acid. Compounds were identified by retention times and multiple reaction monitoring. Quantification was relative to deuterated internal standards.

**Xylem sap ABA, ABA-GE and phaseic acid**

Immediately after determining $\psi_{leaf}$, xylem sap was extracted from the same leaf to determine ABA, ABA-GE and phaseic acid. The extraction was achieved by applying a constant overpressure (+200 psi for well-watered and +300 psi for water deficit stressed), to the leaf in the pressure chamber. This forced more xylem sap to exude from the cut leaf petiole. The exuded xylem sap (and possibly apoplastic fluid, for a few water deficit stressed plants) was carefully pipetted into pre-weighed Eppendorf tube. Sap weight was recorded before tubes were snap-frozen in liquid nitrogen and stored at -80°C until analysis. Xylem sap were dried and analysed for ABA, ABA-GE and phaseic acid using the same method for leaf tissue analysis described earlier.

**Experiment II – canopy temperature, lint yield and fibre quality of high N furrow irrigated cotton crops**

In the 2015/2016 Australian cotton season, seeds of cotton (Sicot 71 BRF) were sown on experimental plots at ACRI. Plots were supplied three levels of N: 80, 160 and 240 kg N $ha^{-1}$ prior to sowing. Experiment II was sown in a randomised complete block design with four blocks. Each treatment plot had eight rows of raised beds. Beds were 10 m long and separated by 1 m furrows. Treatments within a block were 2 m apart. For irrigation, furrows were flooded when there was a soil moisture deficit of 70 mm at a depth of 1m compared against a pre-season determined full-point. Soil moisture deficit was determined using neutron moisture meters.

Measurement of Tc started at 56 DAS, 7 d after the crop reached first square i.e. 50% of plants had one square. This was done using ARDUCrop infra-red thermometers placed either in the 4th or 5th row of each treatment plot. A total of 12 ARDUCrop infra-red thermometers were used to continuously measure Tc. In addition to Tc, weather was monitored using a weather station at the research field. Measurements of Tc continued until 150 DAS during which the crop was irrigated six times. Of importance were three irrigation events spanning 23-day period at flowering, then 12-day period post-flowering, and 20-day period at boll opening. Irrigation was terminated at crop maturity (148 DAS) to inhibit new growth, in preparation for harvest.

Lint yield and fibre quality were determined for each treatment plot at crop maturity. Lint yield (kg $ha^{-1}$) was based on machine harvests, using a spindle picker (modified Case IH 1822), from one 10 m row (4th row) per plot. The harvested seed cotton was ginned in a 20-saw gin with a pre-cleaner (Continental Eagle, Prattville, AL, USA). 250 g subsamples o

harvested seed cotton were analysed for fibre quality including fibre length (mm), micronaire (unitless), strength (cN $Tex^{-1}$), elongation (fibre extension before breaking, %), uniformity (the ratio of the average fibre length to the average of the longer half) and short fibre index (%). Fibre quality was tested using a High Volume Instrument (HVI) model 1000 (USTER® Technologies Inc., Charlotte, NC). Crop management followed best agronomic practices established at ACRI.

**Statistical analysis**

For Experiment I, volumetric soil moisture was analysed by ANOVA with water, nitrogen and their interactions as factors. Sampling time was added as a factor. Plant growth (height, leaf area and above-ground biomass), $\psi_{leaf}$, leaf level gas exchange, and concentrations of ABA, ABA-GE and phaseic acid in leaves and xylem sap, were analysed by residual maximum likelihood (REML) in Genstat (GenStat® 18th Edition, VSN International Ltd. UK).

Mean daily maximum Tc between watering/irrigation events for Experiment 1 (two 7-day stress period at flowering) and Experiment II (a 23-day period at flowering, a 12-day period post-flowering, and a 20-day period at boll opening) obtained from generalised additive models (GAM, described below) were also analysed by REML. Water, nitrogen, stress period and their interactions were treated as fixed terms, experimental unit as the random term. For experiment II, the effect of nitrogen on lint yield and fibre quality were analysed by one-way ANOVA after tests for normality and homogeneity of variance. Replicate was treated as blocking factor.

Canopy temperature for each stress period (two for Experiment I and three for Experiment II) was modelled using the gam function in the package "mgcv", Wood (2011) in R (ver. 3.1.3; R Development Core Team, 2015). Day was fitted as a covariate to model any linear trends in the data and both a sin and cosine term was included to model the overall diurnal pattern of Tc. Adding a spline term accounts for day-to-day smooth variation in Tc. Adding a plot term and the interaction of plot with all other terms fits a curve for each plot that comprises three components, linear trend plus overall diurnal pattern, plus additional smooth variation.

## RESULTS

Growth conditions in the glasshouse during Experiment I were similar to that of the field in Experiment II, with mean temperature of 25°C (SD±6) and 26°C (±8) for Experiment I and II, respectively. Mean daily vapour pressure deficit (VPD) was 1.24 and 1.70 kPa for experiments I and II, respectively.

### Soil moisture and leaf water potential

Volumetric soil moisture in Experiment I was highest for control-N pots and decreased with increase in nitrogen for both well-watered and water-stressed plants (Table 1). Soil moisture for well-watered plants was three to four times more than that of water-stressed plants.

There was a significant water, N and sampling time interaction effect on $\psi_{leaf}$ for Experiment I (Table 1). Under well-watered conditions there was no significant nitrogen effect on $\psi_{leaf}$ at 70 and 86 DAS. Mean $\psi_{leaf}$ decreased from -1.34 MPa at 70 DAS to -1.62 MPa at 86 DAS. Leaf water potential of water deficit stressed plants decreased from -2.13 MPa in control-N plants to -3.47 MPa in 200 N and to -3.73 MPa in 300 N at 70 DAS. There

was no difference at the end of the second water stress regime (at 86 DAS) between control-N (-2.62 MPa) and 300 N (-2.63 MPa) plants, although they both differed significantly from 200 N (-2.95 MPa) plants. Mean $\psi_{leaf}$ at 86 DAS for water deficit stressed plants was -2.73 MPa.

**Canopy temperature**

In Experiment I, high nitrogen plants under well-watered condition had a cooler canopy than the control-N plants, while under water stress high nitrogen plants had warmer canopies relative to the control-N plants (Figure 1, Table 2). The nitrogen and water interaction term for daily maximum Tc was significant ($P$=0.003). This effect of nitrogen on Tc was consistent in warm and high VPD conditions: conditions that resulted in Tc exceeding the optimum for cotton photosynthesis and yield (i.e. 28–29°C; Conaty et al., 2012). This was the case in Experiment I as well as II (Table 2).

In Experiment I, the difference in daily maximum Tc between well-watered high nitrogen (200 and 300 N) and control-N plants increased with time. During the first 7-day cycle control-N and 200 N Tc differed by -0.4 to 0.9°C but this difference increased to -0.8 to -1.3°C by the second water-stress period (compare Figs. 1a with 1b). Similarly, increase in Tc with time was observed among water deficit stressed plants (Figure 1c, d). For example, between control-N and 300 N, the difference was from 0.2 to 0.7°C during the first stress period and from 0.7 (excluding day two) to 1.2°C during the second stress. Overall, mean daily maximum Tc of well-watered plants was ~2°C cooler (at 29°C ±0.2) than water-stressed plants (at 31°C ±0.2).

As in Experiment I, under water deficit stress conditions in the field (Experiment II) high nitrogen crops had warmer canopies than the control-N crop (Figure 2). The difference in

daily maximum canopy temperature between irrigations at first flower were on average 0.7 (SEM±0.1) and 0.8°C (±0.1) warmer for crops treated with 160 and 240 kg N $ha^{-1}$, respectively, than the control-N crop (Figure 2a). Post-flowering this difference reduced to 0.4°C (±0.0) for both high nitrogen crops and was insignificant during boll opening being 0.1(±0.0) and 0.3°C (±0.0) for 160 and 240 kg N $ha^{-1}$, respectively (Figure 2b, c).

**Plant growth (height, leaf area and above-ground biomass)**

In Experiment I, plant height was slightly higher for well-watered plants than water-stressed plants, increasing with nitrogen supplied and sampling time (Figure 3a, b). Both leaf area and above-ground biomass had significant interaction effects of water, nitrogen and sampling time ($P<0.05$; Figure 3c, d for leaf area; Figure 3e, f for above-ground biomass). Plant growth until 43 DAS, prior to imposing nitrogen or water stress, was uniform. The application of the nitrogen treatment to the 200 and 300 N plants at 60 DAS resulted in significant growth as soon as five days after application of N (data not shown). This difference in growth was still apparent in well-watered plants by 86 DAS but only until 70 DAS in water-stressed plants. (Figure 3). At 86 DAS, high nitrogen well-watered plants were larger than control-N well-watered plants. High nitrogen well-watered plants were taller (plant height increased 18 to 22%), had greater canopy cover (27 to 39% more leaf area), and accumulated more dry mass (21 to 33% more biomass). In contrast, high nitrogen water-stressed plants were not significantly larger than control-N plants (Figure 3). For control-N plants, there were no significant differences between well-watered and water deficit stressed plants in height, leaf area and above-ground biomass.

**Leaf-level gas exchange**

The effect of water and nitrogen interaction was significant for stomatal conductance ($P$=0.034), marginal for photosynthesis ($P$=0.052) and not significant for transpiration ($P$=0.487) in Experiment I (Figure 4). The significant interaction of water and nitrogen for stomatal conductance was driven by the main effect of water ($P$<0.001, Wald statistic/degree of freedom=275.26) not nitrogen ($P$=0.10, Wald statistic/d.f.=4.56). For photosynthesis, main effects of water and nitrogen were both significant ($P$<0.001 for water and $P$=0.005 for nitrogen) whereas for transpiration only the main effect of water was significant ($P$<0.001). Well-watered plants supplied high nitrogen had marginally higher stomatal conductance and greater photosynthesis than the control-N plants but similar rates of transpiration (Figure 4). Under water deficit stress, high nitrogen plants had reduced stomatal conductance compared with the control-N plants (Figure 4a, b). Photosynthesis of water-stressed plants decreased with increase in nitrogen supplied at first sampling but had no significant response to nitrogen at second sampling (Figure 4c, d). Change in transpiration of water-stressed plants to nitrogen and sampling time mirrored that of stomatal conductance (compare Figure 4e, f with 4a, b). Also, there was a gradual trend of reduced photosynthesis, stomatal conductance and transpiration with increase in nitrogen but only at 69 DAS. Gas exchange characteristics of water-stressed plants were a fraction of those of well-watered plants, being about one-sixth for stomatal conductance, one-third for photosynthesis and one-quarter for transpiration. All other interaction terms were not significant, except water and sampling time for photosynthesis.

**Leaf level water use efficiency**

Leaf level WUE was taken as transpiration ratio, calculated as the quotient of leaf carbon assimilation at saturated light conditions and transpiration. Water use efficiency did not differ significantly ($P$=0.10) with N or the interaction of N with other terms, but it did with water ($P$<0.001), sampling time ($P$<0.001) and their interaction ($P$=0.045). At first sampling, mean WUE of well-watered plants at 3.38 μmol $CO_2$ $mol^{-1}$ of $H_2O$ (SEM±0.31) was lower ($P$<0.001) than those of water deficit stressed plants at 8.00 μmol $CO_2$ $mol^{-1}$ of $H_2O$ (±1.31). Mean WUE at second sampling were much lower but they were similarly so, in that WUE for well-watered plants at 2.56 μmol $CO_2$ $mol^{-1}$ of $H_2O$ (±0.09) was lower ($P$<0.001) than those of water deficit stressed plants at 4.63 μmol $CO_2$ $mol^{-1}$ of $H_2O$ (±0.23).

**Leaf ABA, ABA-GE and phaseic acid**

Generally, well-watered plants had less leaf ABA, ABA-GE, and phaseic acid than water deficit stressed plants (Figure 5). There were significant water, nitrogen and sampling time effects on leaf ABA, ABA-GE, and phaseic acid (Figure 5). Well-watered plants in Experiment I showed no significant change in leaf ABA with nitrogen or sampling time. Similarly, leaf ABA-GE of well-watered plants did not respond to nitrogen supply but it increased with time. Like leaf ABA of well-watered plants leaf phaseic acid did not respond to nitrogen or change with time.

On the other hand, under water deficit stress high nitrogen increased leaf ABA and ABA-GE at first sampling (Figure 5a, c) but no nitrogen effect was observed at second sampling (Figure 5b, d). The only leaf phytohormone which was greater in high nitrogen plants than in control-N plants at both first and second sampling was phaseic acid (Figure 5e, f).

**Xylem sap ABA, ABA-glucose ester and phaseic acid**

Significant second order interactions of water, nitrogen, and sampling times were observed for xylem ABA, ABA-GE, and phaseic acid in Experiment I. The concentration of xylem sap ABA neither responded to nitrogen nor sampling time in well-watered plants but in water-stressed plants it increased with increase in nitrogen at first sampling alone (Figure 6a, b). Xylem sap ABA-GE of well-watered plants showed an increasing trend with increase in nitrogen supply, albeit insignificant, at first sampling and then a reduction with increase in nitrogen at second sampling. A similar trend was observed in water-stressed plants; however, the changes were significant. Xylem sap ABA-GE increased with nitrogen supply at first sampling and decreased at second sampling with additional nitrogen supply (Figure 6c, d). Changes in xylem sap phaseic acid concentration of well-watered and water-stressed plants in response to nitrogen supply and sampling time mirrored those of xylem sap ABA (Figure 6e, f). Well-watered plants had a lower concentration of xylem sap ABA, ABA-GE and phaseic acid compared with the water deficit stressed plants irrespective of sampling time, except for ABA-GE at second sampling (Figure 6). Under the water deficit stressed condition, increase in nitrogen was accompanied by increased xylem sap ABA, ABA-GE, and phaseic acid at first sampling, but at second sampling xylem sap ABA, ABA-GE, and phaseic acid were least in 300 N plants (Figure 6).

**Experiment II lint yield and fibre quality**

Under field conditions (Experiment II) lint yield and fibre quality did not differ significantly between treatments (Table 3). Mean lint yield was 2978 kg $ha^{-1}$ and mean fibre length, micronaire, strength, elongation, uniformity and short fibre index were 29.7 mm, 4.21, 28.26 cN $Tex^{-1}$, 5.08 % (±0.06), 81.8, and 7.5% respectively.

## DISCUSSION

We demonstrated that high nitrogen influences cotton Tc. In the glasshouse, under well-watered and water deficit stress, high nitrogen enhanced plant growth (leaf area, above-ground biomass, and height), increased demand for water (marked by reduced soil water), altered gas exchange processes (stomatal conductance, photosynthesis and transpiration) and plant water status (i.e. $\psi_{leaf}$). Under water deficit stress, simultaneous changes in leaf and xylem sap ABA concentration were also observed. In the field, under furrow-irrigated growing conditions plants supplied higher/excessive nitrogen, marked by no difference in lint yield and fibre quality, had warmer Tc.

### High nitrogen stimulated growth and altered leaf gas exchange with subsequent effect on canopy temperature.

Under glasshouse conditions, increase in nitrogen supply stimulated growth. High nitrogen plants had larger leaf area, accumulated more biomass and were taller (Figure 3). These larger plants had a greater demand for water, with 14–21% increase in stomatal conductance to water vapour (Figure 4). Consequently, the bigger plants under the water deficit stress condition more rapidly utilised stored soil moisture (Table 2), exposing plants to water stress conditions. This water stress condition was indicated by reduced stomatal conductance and photosynthesis, lower $\psi_{leaf}$, accumulation of ABA in leaf and xylem sap and a higher Tc, when compared to the control (Figs 4-6, Table 2). The increase in nitrogen supply presented no gain in terms of leaf-level WUE. Bigger plants, which used more water, were not more efficient in water use either under water stress or well-watered conditions.

In addition to enhancing growth increased nitrogen supply also affected leaf-level gas exchange differently under well-watered and water deficit stressed conditions (Figure 4). Under well-watered condition, increases in stomatal conductance and photosynthesis was probably due to leaf nitrogen increasing with greater nitrogen availability. Leaf nitrogen is positively correlated with stomatal conductance (Franks and Beerling, 2009; Xiong et al., 2015), and high leaf nitrogen translates into greater investment in metabolic processes (especially photosynthesis, Terashima and Evans, 1988; Evans, 1989) and plant structural properties (Harrison et al., 2009). Under well-watered conditions high nitrogen supply increased investment in both structural properties (in terms of leaf tissue, see Figure 3) and photosynthesis (Figure 4). However, under water deficit stress the effects of increased nitrogen supply were limited to structural properties (i.e. formation of leaf tissues, Figure 3). When water deficit cycles were imposed on plants with increased nitrogen supply, their larger plant canopy and above-ground biomass resulted in a relative increase in water demand, leading to more extreme water limitations. This resulted in restrictions in stomatal conductance, which limited evaporative cooling and thus a warmer Tc. We acknowledge that the water stress imposed in the glasshouse does not fully represent conditions experienced in the field, including, for example, the absence of wetting and drying cycles. As such plants in the glasshouse may not have acclimated to the water stress imposed. In scenarios where water stress is a sustained or primary limitation to crop production, then plant growth and canopy size will not be as large, thus plants will not be as susceptible to water stress due to increased plant growth as a result of high nitrogen supply.

**Accumulation of ABA in bulk leaf tissues influences Tc of water stressed cotton plants supplied high nitrogen.**

Cotton plants exhibited common physiological responses to water-deficit in terms of ABA accumulation, regulation of stomatal conductance and influence on Tc (Radin and Ackerson, 1981; Radin et al., 1982). Leaf and xylem sap ABA, ABA-GE and phaseic acid accumulated under water stress. Similar responses have been observed in other crops (Ali et al., 1998; Tardieu et al., 1992). The accumulation of ABA reduced stomatal conductance by causing guard cells to close (Pantin et al., 2013), consequently increasing Tc. Increasing nitrogen supply amplified the accumulation of ABA, ABA-GE and phaseic acid. This agrees with a recent study (Li et al., 2017), which showed consistently higher levels of xylem sap ABA in water stressed cotton plants grown on more soil nitrogen. These responses of ABA and stomatal conductance to high nitrogen in water stressed cotton contributed to warmer cotton Tc of plants supplied higher nitrogen.

Concentration of bulk leaf tissue and xylem sap ABA were similar to previous reports for cotton (Dong et al., 2008; Radin et al., 1982). The abundance of ABA, ABA-GE and phaseic acid were significantly higher in the leaves than in the xylem sap of non-stressed plants. For example, bulk leaf tissue ABA of water-stressed plants was at least 50% greater than xylem sap ABA. It has been suggested that xylem sap ABA has better control of stomatal conductance than bulk leaf tissue ABA (Saradadevi et al., 2016) because it is thought that the effect of ABA on stomatal conductance is driven by the accumulation of apoplastic ABA (Sirichandra et al., 2009). However, we observed greater concentration of ABA, ABA-GE and phaseic acid in bulk leaf tissue than in xylem sap. This suggests that ABA biosynthesis and catabolism were localised, with little or no response to the relatively smaller quantity of

xylem sap ABA transported from other sources (e.g. root) via the xylem to the leaf. Some studies with other crops support this, with stomatal conductance correlating better with leaf ABA (Henson et al., 1989) and poorly with xylem sap ABA (Ali et al., 1998; Atkinson et al., 1989).

**Bulk leaf tissue ABA-GE and phaseic acid probably had minutiae effect on stomatal**

Bulk leaf tissue ABA-GE and phaseic acid probably had minutiae effect on stomatal conductance and ultimately Tc, although they both increased with water deficit stress and increase in nitrogen supplied (at first sampling for ABA-GE and both sampling times for phaseic acid). This is because conjugation of ABA with glucose to form ABA-GE is considered an irreversible process that sequesters ABA from cells (Zeevaart and Creelman, 1988). Similarly, the presence of phaseic acid indicate activities of metabolic pathways functioning to rid cells of high ABA.

**Observation of field cotton crops confirms glasshouse study.**

Our field experiment data provided additional support for a link between high nitrogen supply and Tc of furrow grown cotton crop. In the absence of initial soil nitrogen data, but of yield data that did not significantly increase with additional nitrogen supply, it is likely that the soil had high levels of residual nitrogen prior to nitrogen treatment application. Hence, even the 80 kg N $ha^{-1}$ treatment was still high yielding. These potentially high and excessive nitrogen treatments combined with differences in canopy temperature under irrigated field conditions suggests cotton Tc is amenable to high nitrogen availability.

Canopy temperature of furrow irrigated crops with higher nitrogen application rates (160 and 240 kg N $ha^{-1}$) at ACRI were warmer by on average 0.7°C during peak flowering than the

reference crop (80 kg N $ha^{-1}$) (Figure 2). This difference in temperature was similar to that recorded under glasshouse conditions, among similarly developed plants. However, it was tempered by 68.6 mm of rain at the start of peak flowering and 100.4 mm of rain between the second and third irrigation events post-flowering, which resulted in a cooler and lower VPD growth environment. A larger difference in Tc between control and high nitrogen crops might have been observed under different environmental conditions e.g. lower or no rainfall, higher VPD and solar radiation.

**Implications for Tc-based cotton production system**

In the context of Tc based irrigation scheduling that utilise a temperature-time threshold combination to schedule irrigation events, high nitrogen crops may exceed the temperature threshold earlier. However, additional studies need to determine if high nitrogen applications will trigger more frequent irrigation events, as the time factor remains unstudied. However, it is feasible to suggest that as high nitrogen levels result in larger plants with larger canopies, an increase in plant water demand may result in more frequent furrow irrigation events. If crops are irrigated to ensure water supply does not limit yield, this increased plant water demand and consequent increase in the frequency of irrigation events could result in greater overall water use within a season. This is of particular concern under scenarios where an increase in biomass does not result in a concurrent gain in lint yield and quality. In our field experiment, where no gain in yield or quality with increased nitrogen application was the case, indicating supra-optimal nitrogen supply, we still recorded significant influence of high nitrogen in cotton Tc.

In our field study, lint yield of the control crop was higher than the Australian cotton industry average (2200 kg $ha^{-1}$) for the last decade (Roth et al., 2013), and similar to average

yield of best practice cropping system at ACRI for the previous three seasons (2930 kg $ha^{-1}$). Lint quality of the high nitrogen crop was not different from that of the control-N crop. Irrespective of nitrogen supplied, lint quality was within the desired range for micronaire (3.8 to 4.5), close to the target fibre length of Australian breeding projects (32 mm) and similar to that of premium fibre grown under the same environmental conditions for other fibre quality characteristics (Clement et al., 2012, 2014; Constable et al., 2015).

**Conclusion and recommendations for future studies**

Nitrogen interacts with plant water status to influence cotton Tc. High nitrogen plants under water deficit conditions had warmer Tc. These results have implications for the Australian furrow irrigated cotton system, which is beginning to adopt the Tc based irrigation scheduling system. We recommend that future studies should investigate whether the cause of the observed increased Tc is solely due to increased plant water demand associated with larger plants. This could be achieved by assessing if the optimum temperature for cotton physiological functions is influenced by nitrogen supply. However, we suggest that as Tc based irrigation scheduling is based directly on the plant's water requirement, a Tc irrigation scheduling approach that utilises a temperature-time threshold will ensure plant water status and productivity is maintained to an optimum. In addition, future field studies should confirm if the lack of lint yield and fibre quality response to nitrogen is associated with increased plant biomass and water use.

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## CONFLICTS OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure 1. Relative change in mean daily maximum canopy temperature (Tc) of glasshouse-grown plants (Experiment I) supplied with extra 200 (grey bars) or 300 (black bars) kg N $ha^{-1}$ compared with control-N plants. Plants were either kept well-watered (a, b) or exposed to water deficit stress (c, d) during the periods 64 to 70 (right panels) and 80 to 86 (left panels) days after sowing.

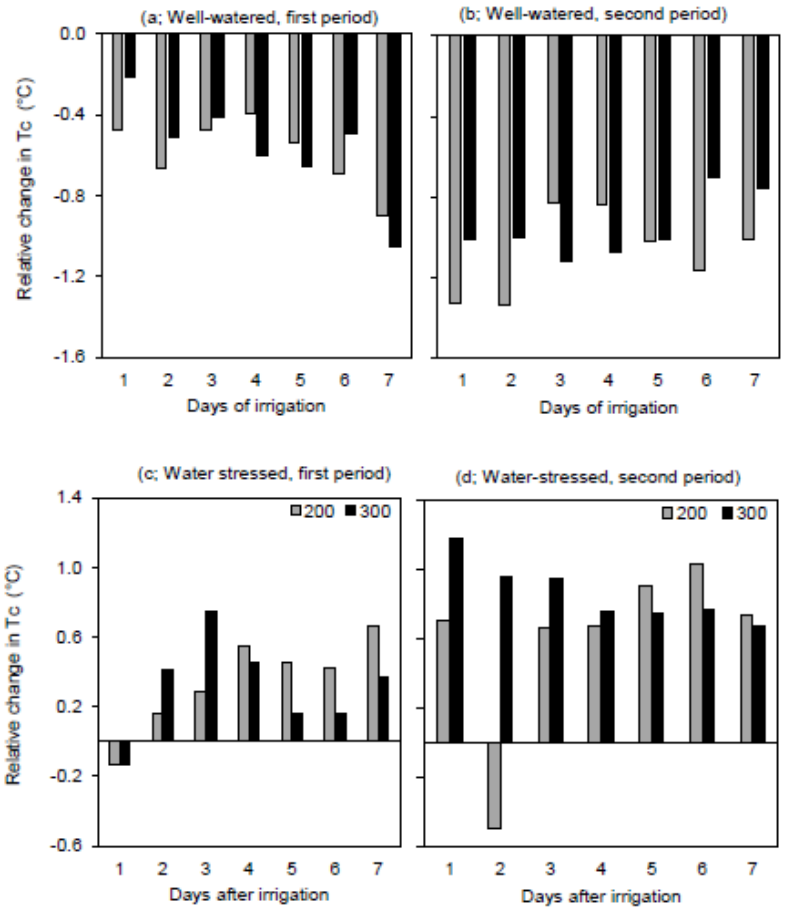

Figure 2. Relative change in daily maximum canopy temperature (Tc) of field-grown cotton (Experiment II) supplied with 160 kg N $ha^{-1}$ (grey circles) or 240 kg N $ha^{-1}$ (black circles) compared to those supplied 80 kg N $ha^{-1}$ during the 2015/2016 growing season at ACRI, Narrabri. Each panels represent the time between irrigations beginning at first flower (a) through boll formation and opening (b, c).

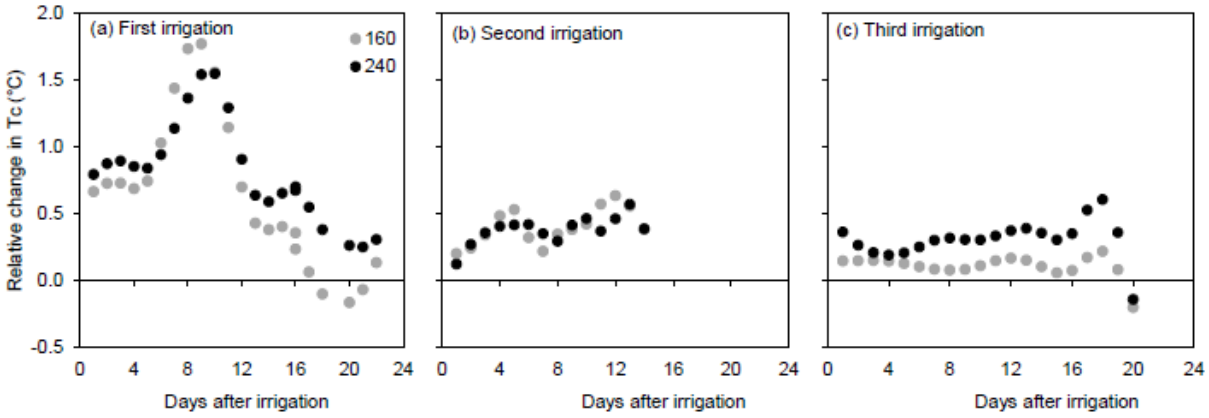

Figure 3. Plant height (a, b), total leaf area per plant (c, d), and above-ground biomass (dry mass, DM; e, f) of glasshouse-grown plants (Experiment I) under three nitrogen levels in either well-watered (watered) or water-stressed (stressed) condition. The nitrogen levels were control (white bars), 200 (grey bars) or 300 (black bars) kg N $ha^{-1}$. Results presented are for the end of the first water-stress period (left panels; 70 DAS) and at the end of the second water stress period (right panels; 86 DAS). Bars are means ($n$=12) ±SEM (as error bars).

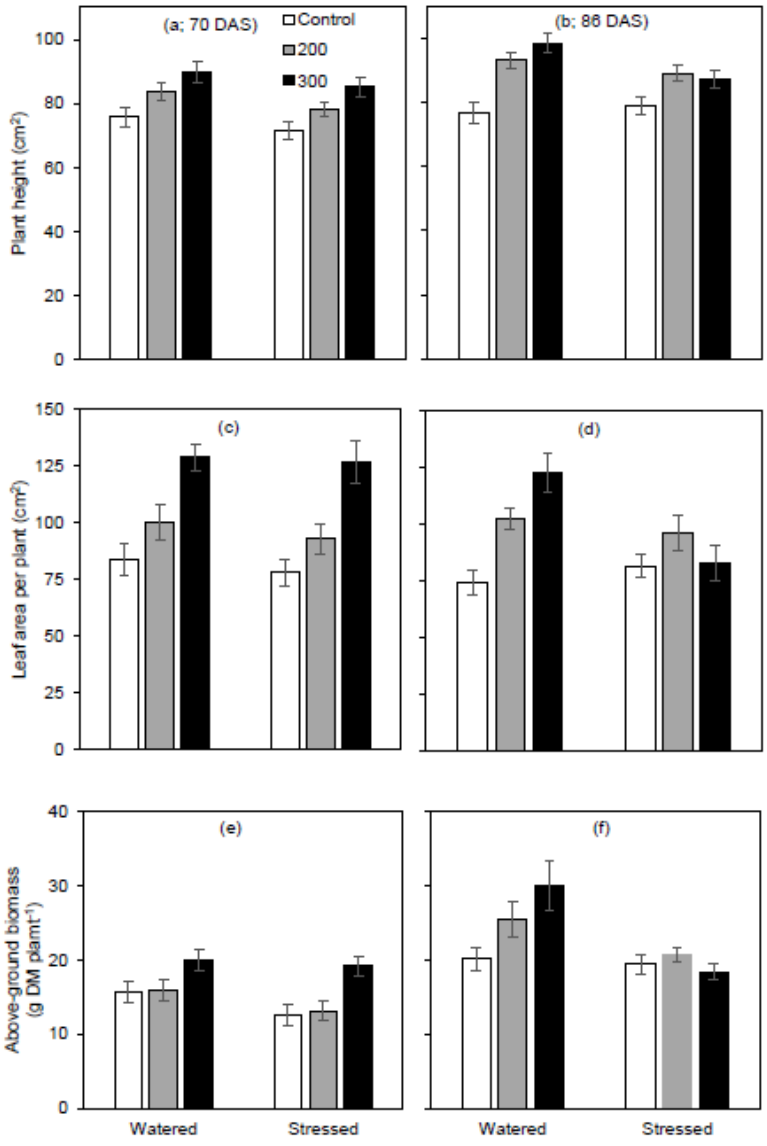

Figure 4. Leaf-level stomatal conductance to water (a, b), photosynthesis (c, d) and transpiration (e, f) of water-stressed (stressed) and well-watered (watered) glasshouse-grown plants (Experiment I) supplied three levels of nitrogen (control, 200 and 300 kg N $ha^{-1}$). Measurements were taken at 69 (left panels) and 85 (right panels) days after sowing (DAS). Bars are means ($n$=6) ±SEM (as error bars).

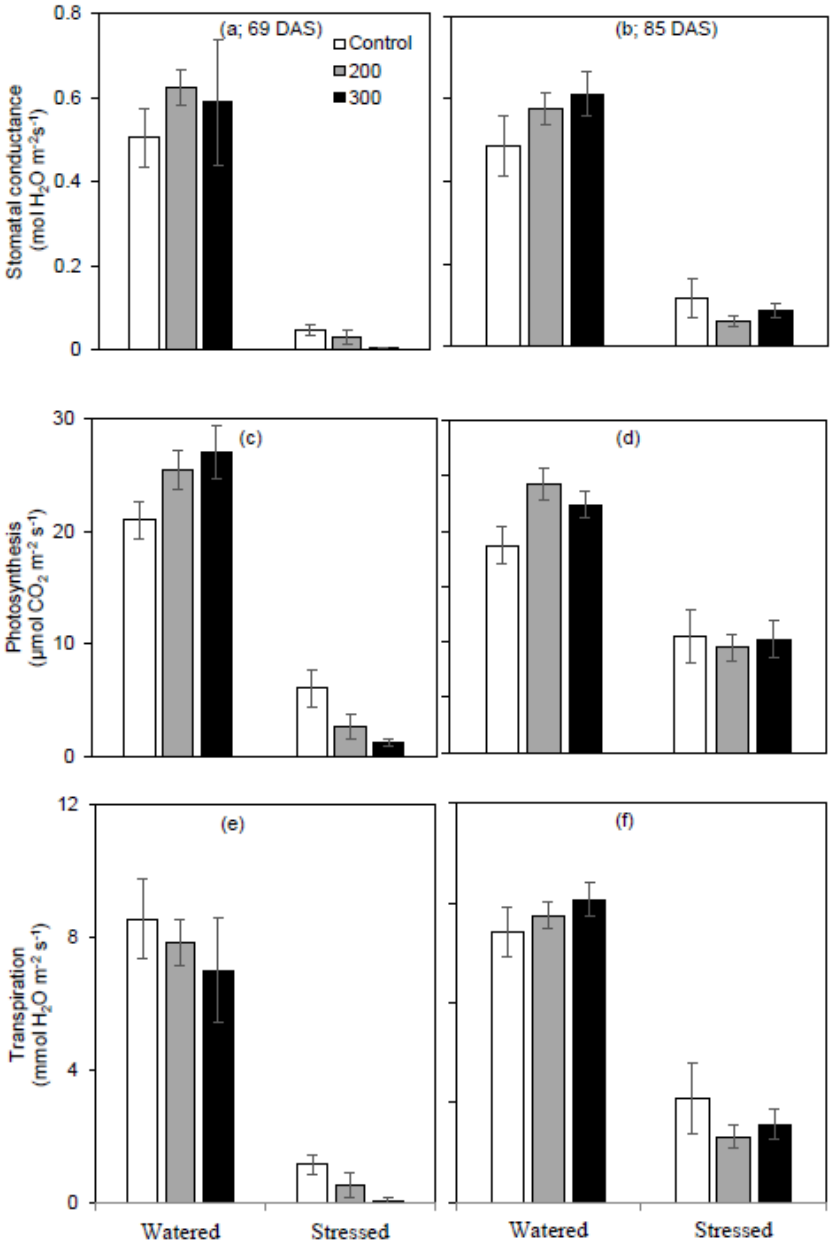

Figure 5. Leaf abscisic acid (a, b), abscisic acid glucose ester (c, d), and phaseic acid (e, f) concentration of glasshouse-grown plants (Experiment I) supplied three levels of nitrogen (control, 200 and 300 kg N $ha^{-1}$) and grown under either well-watered (Watered) or water-deficit stress (Stressed) conditions. Samples were collected on 70 (left panels) and 86 (right panels) days after sowing (DAS). Bars are means ($n$=3) ±SEM (as error bars).

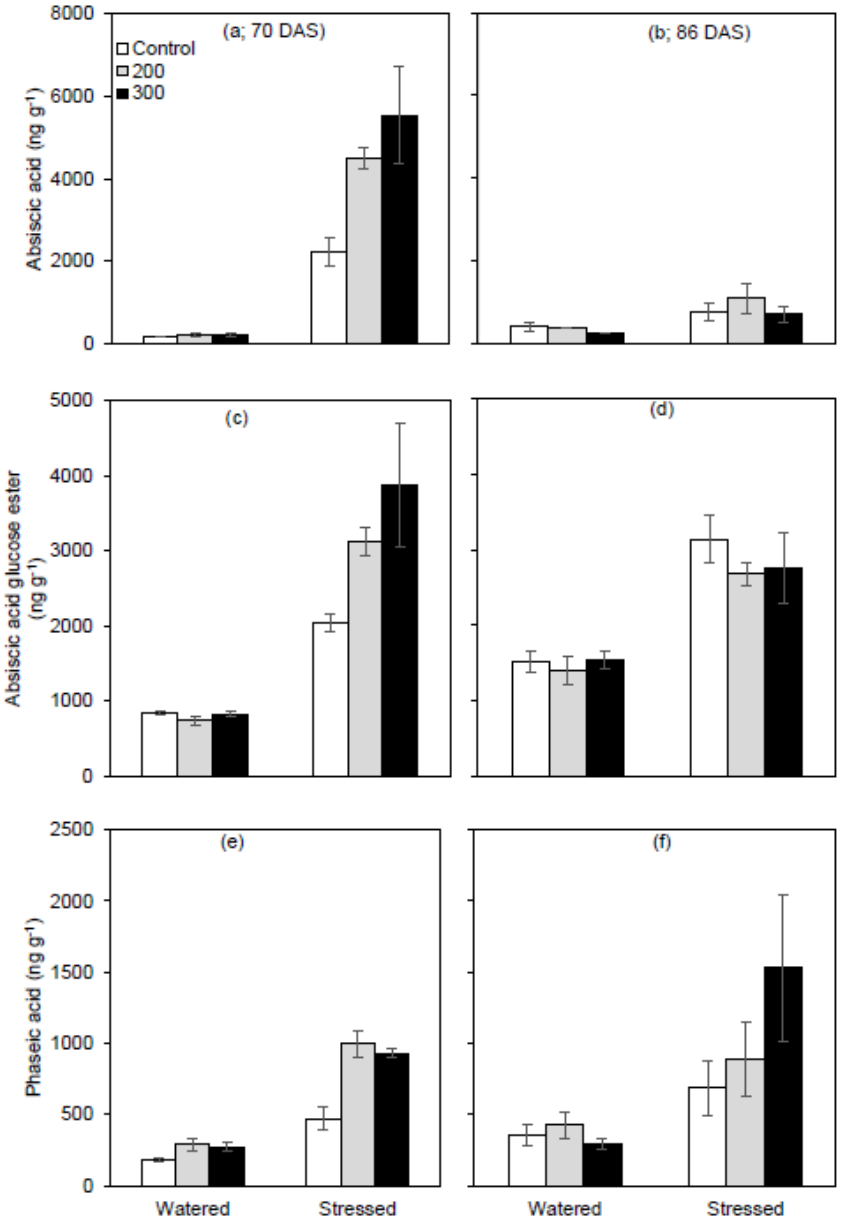

Figure 6. Xylem sap abscisic acid (a, b), abscisic acid glucose ester (c, d), and phaseic acid (e, f) concentration of glasshouse-grown plants (Experiment I) supplied three levels of nitrogen (control, 200 and 300 kg N $ha^{-1}$) and grown under either well-watered (Watered) or water-deficit stress (Stressed) conditions. Samples were collected on 70 (left panels) and 86 (right panels) days after sowing (DAS). Bars are means ($n$=3) ±SEM (as error bars).

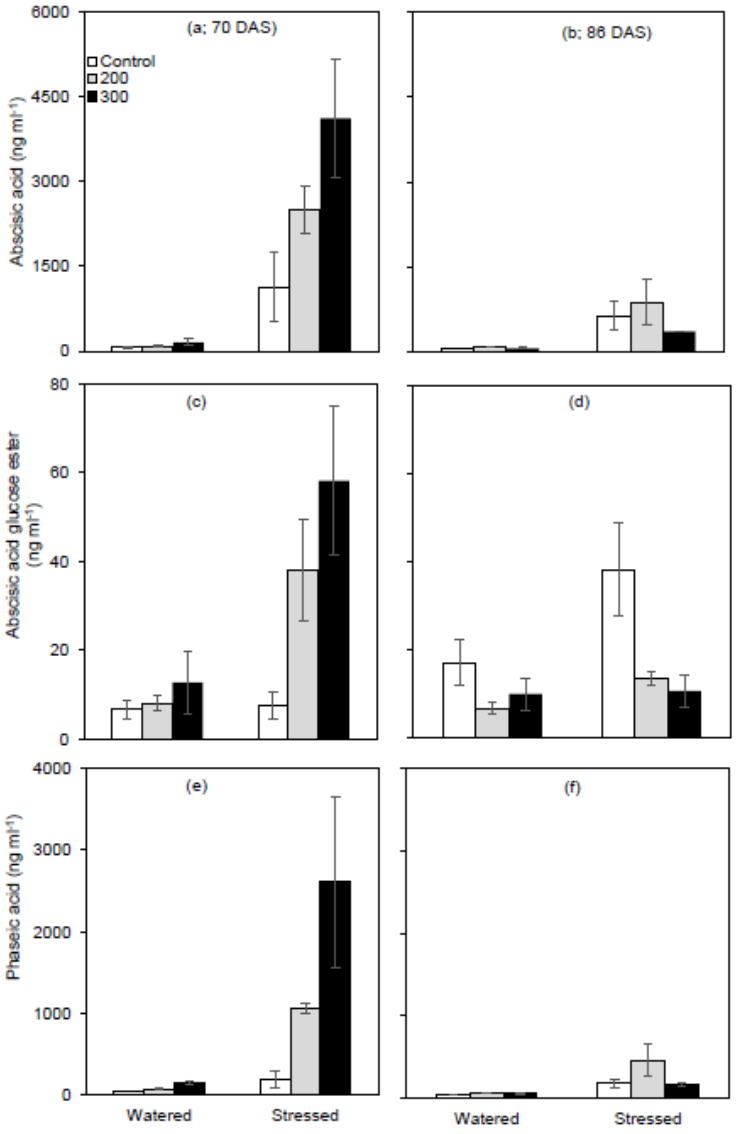

**Table 1. Volumetric soil moisture content and leaf water potential ($\psi_{leaf}$) of well-watered and water deficit stressed glasshouse-grown cotton (Experiment I) at three levels of nitrogen. Data were collected at 70 and 86 days after sowing (DAS) after seven consecutive days of stress.**

| Time | Nitrogen | Volumetric soil moisture[†] % | | $\psi_{leaf}$ MPa | |
|---|---|---|---|---|---|
| | | Well-watered | Water deficit stressed | Well-watered | Water deficit stressed |
| 70 DAS | | | | | |
| | Control-N | 32.1±1.3 | 12.0±1.1 | -1.40 | -2.13 |
| | 200 N | 30.8±1.0 | 8.7±0.6 | -1.32 | -3.47 |
| | 300 N | 25.5±1.6 | 8.1±1.1 | -1.30 | -3.73 |
| | **Mean** | **29.5±1.0** | **9.5±0.9** | **-1.34** | **-3.11** |
| 86 DAS | | | | | |
| | Control-N | 30.3±0.9 | 12.5±1.9 | -1.58 | -2.62 |
| | 200 N | 28.4±1.3 | 8.2±1.9 | -1.65 | -2.95 |
| | 300 N | 26.3±1.6 | 6.3±1.3 | -1.63 | -2.63 |
| | **Mean** | **28.3±1.4** | **9.0±1.6** | **-1.62** | **-2.73** |
| $LSD_{(0.05)}$ | | | | | |
| Water | | 1.9*** | | 0.19*** | |
| Nitrogen | | 2.4** | | 0.23** | |
| Time | | 1.9* | | $0.19^{ns}$ | |
| Water x Nitrogen | | 3.2* | | 0.33*** | |
| Water x Time | | 2.7*** | | 0.27*** | |
| N x Time | | $3.3^{ns}$ | | 0.33* | |
| Water x Nitrogen x Time | | $4.7^{ns}$ | | 0.46** | |

**[†]Means are ± SEM ($n$=24); ns, not significant ($P$>0.05); *, $P$<0.05; **, $P$<0.01; ***, $P$<0.001.**

**Table 2. Mean daily maximum canopy temperature of glasshouse-grown cotton plants (Experiment I) and field-grown plants (Experiment II). Values are averages for the two 7-day cycles of Experiment I and whole flowering period of Experiment II.**

| Water | Nitrogen | Mean daily maximum canopy temperature[†] °C | | |
|---|---|---|---|---|
| | | I (Glasshouse) | | II (Field) |
| Well-watered | | | | |
| | Control-N | 30.0±0.3 | | |
| | 200 N | 29.2±0.3 | | --- |
| | 300 N | 29.2±0.3 | | --- |
| | **Mean** | **29.5±0.2** | | |
| Water-deficit stressed | | | **Nitrogen** | |
| | Control-N | 30.9±0.3 | 80 | 31.1±0.3 |
| | 200 N | 31.4±0.4 | 160 | 31.9±0.5 |
| | 300 N | 31.5±0.4 | 240 | 31.7±0.3 |
| | **Mean** | **31.3±0.2** | | **31.6±0.2** |

[†]**Means are ±SEM; *n*=3 for Experiment I, *n*=4 for field Experiment II.**

**Table 3. Mean yield and fibre quality of field-grown cotton (Experiment II).**

| | Lint yield | Length | Micronaire | Strength | Elongation | Uniformity | Short fibre index |
|---|---|---|---|---|---|---|---|
| | kg ha$^{-1}$[†] | mm | | cN Tex$^{-1}$ | % | | % |
| Nitrogen (kg N ha$^{-1}$) | | | | | | | |
| 80 | 2869[a] | 29.3[a] | 4.26[a] | 28.40[a] | 5.20[a] | 81.4[a] | 7.7[a] |
| 160 | 3110[a] | 29.8[a] | 4.10[a] | 28.43[a] | 4.95[a] | 82.0[a] | 7.6[a] |
| 240 | 2955[a] | 29.9[a] | 4.26[a] | 28.25[a] | 5.08[a] | 81.9[a] | 7.3[a] |
| Mean | **2978[a]** | **29.7** | **4.21** | **28.26** | **5.08** | **81.8** | **7.5** |
| $LSD_{(0.05)}$ | 291 | 0.9 | 0.12 | 0.97 | 0.33 | 0.9 | 1.3 |

[†]**Numbers in a column with same alphabet are not significantly different (*P* > 0.05);**

***n*=12**