1700 Simple electrolyte leakage protocols to detect cold tolerance in cotton genotypes

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Temperature is the dominant environmental factor affecting cotton development and yield. Chilling temperatures can significantly reduce cotton yield. The ability to screen cotton for cold tolerance can lead to the development of cultivars that can maintain yield and quality in marginal cotton growing regions where exposure to chilling stress is more common. This study tests the hypothesis that the relative electrical conductivity (REC) technique can be used in assays to detect cold tolerance in cotton. A cold water bath protocol was developed using various cold temperatures (3, 0, -2, -5°C) and a control (20°C) for 2 and 4 h. The 0°C for 2 h assay gave the best discrimination in REC between two cultivars, Namcala and DP16. A complementary whole plant assay was also developed using various night temperatures (5, 2 and -1°C) and a control at 20°C for 6 nights, with day temperatures at 30°C. The 5°C for 6 nights assay increased REC and reduced photosynthesis and chlorophyll fluorescence in DP16 compared to Namcala. DP16 plants exposed to -1°C died after 4 weeks while Namcala remained alive. Hence, both assays corroborate showing that Namcala is more cold tolerant than DP16. These newly developed protocols have the potential to be used in breeding programs to screen for cold tolerant cotton breeding lines and cultivars.

Key Words: Cotton, *Gossypium hirsutum*, cold tolerance, electrolyte leakage, genotype

Introduction

Cotton requires a long, hot growing season for maximum production. It is a frost sensitive, short day plant and temperature is the dominant factor affecting growth, development and yield . Sub-optimal (cold) temperatures encountered during the season can decrease developmental rates and reduce yield and fibre quality . Supra-optimal (hot) temperature conditions also have a similar effect . Sub-optimal and supra-optimal temperatures are being experienced more often in cotton production as the industry is expanding into marginal areas including Gunnedah, Bourke and Hillston in Australia.

Australia’s cotton growing season lasts approximately six months, starting in September/October and ending in March/April . The start of the season is governed by the sowing date. Early sowing is important in areas with short growing seasons (marginal areas), provided no chilling injury occurs . Chilling injury remains the greatest restraint, particularly in marginal cotton growing regions . The possibility of cold conditions and frosts make early sowing inherently risky. In cotton exposed to sub-optimal temperatures, germination and emergence is slower, and this often results in greater variability in growth and establishment . These effects will carry through to yield, with crops exposed to cooler temperatures early in the season producing lower yields and fibre with reduced strength and diameter (micronaire) . Overall, plants exposed to low temperatures have increased time to reach developmental stages, and slower accumulation of biomass . Frosts will retard growth of cotton plants or even kill them. Hence, the date of the first frost in autumn determines the end of the season.

Cold shock is thought to occur when plants are exposed to a minimum daily temperature ≤ 11°C . When this occurs, growth and development in the following day can be reduced, regardless of the maximum temperature reached. One cold shock event (i.e. ≤ 11°C) extends the duration to flowering by 5.2 day degrees. Marginal growing areas such as Hillston, Warren and Gunnedah have the highest number of cold shock days which is to be expected as they have shorter growing seasons. Recent research suggested that 12^{o}C is not a suitable temperature threshold to define chilling damage in post-emergent cotton . The original work to define cold shock was carried out using cultivars that are different to the ones grown today . Recent studies suggest that the base temperature for Australian cultivars is closer to 15°C than 12°C .

The ability to screen various cultivars for cold tolerance will benefit the cotton industry. A technique for use in breeding programs should be developed for rapid, reliable and low cost screening. Membrane thermostability has been identified in most modern literature as an indicator of thermo-tolerance. Disruption and damage to cell membranes altered permeability, and resulted in a loss of solutes (electrolytes) . This damage will cause injury to the plant and cell death will result. Electrolyte leakage reflects damage to cellular membranes . The amount of electrolyte leakage is a function of membrane permeability . An increase in electrolyte leakage indicates an increase in membrane permeability and reduced cell tolerance to temperature change.

Previous studies have used relative electrolyte leakage as a measure of cell damage from imposed temperature regimes, in order to identify non-optimal temperature tolerance. Initial studies based on cellular membrane thermostability in cotton have focused on heat and chilling stress There is currently no protocol using electrolyte leakage to distinguish between cold tolerant and sensitive cotton cultivars.

This paper proposes protocols for detecting cold tolerance in cotton using an electrolyte leakage technique. It builds on previous studies , and aims to identify quick and simple assays of cotton physiological performance and yield when exposed to chilling. This paper will test the hypothesis that an electrolyte leakage assay can be used to identify cultivar differences in cold tolerance.

Materials and Methods

The experiment was conducted at Darlington Glasshouse in the University of Sydney, Australia. Seeds of two cotton cultivars, DP16 and Namcala were germinated in the dark at 25ºC. After emergence, the seedlings were sown into 25 cm diameter plastic pots filled with standard commercial potting mix. Four seedlings were sown in each pot. Irrigation was set up to water the pots twice daily at 150 mL. Aquasol® (NPK analysis 23:4:18) liquid fertiliser was applied at 1g/L of water once a week. The pots were arranged in the glasshouse in a completely randomised design with 8 replicates (pots) per cultivar. The glasshouse was set at 30°C/20°C day/night temperature, respectively. Photoperiod was set at 16 h/8 h day/night using glasshouse sodium lamps. Aphids were controlled by regular applications of pest oil and Confidor®, and predatory mites (*Phytoseiulus persimilis*) were released to control spider mites.

Electrolyte leakage assay using water baths

An assay was designed to develop a technique similar to that of Rahman *et al.* that was originally developed to screen cultivars for heat tolerance. The critical temperature to discriminate cold tolerance was not known for cotton and a range of temperatures had to be

experimented with. Plants were grown to the flowering stage and three 10 mm leaf discs were sampled from two young, fully expanded leaves on each plant (4 plants). The leaf discs were placed in separate test tubes with 10 mL de-ionised water and left to stand for 30 minutes. Initial electrolyte leakage (EC) readings were measured and the test tubes were placed in pre-cooled water baths. Ethylene glycol (50%) was added to the subzero temperature water baths to prevent the solution from freezing. Water baths were set at four temperatures (3, 0, -2, -5°C ± 1°C) and a control (20°C ± 1°C) in thermostatically controlled cabinets and checked using thermometers. The test tubes were immersed in the water baths for 2 h and 4 h and EC was measured again. The test tubes were then autoclaved for 15 minutes at 121°C at a pressure of 103kPa and the final EC measurements were taken. Relative electrical conductivity (REC) was calculated as the ratio of the initial EC to the final EC .

Whole plant chilling

Whole plant chilling was conducted to confirm and verify the cold tissue assays. Six replicates (pots) of plants were grown to flowering (13^{th} node stage) and placed in thermostatically controlled growth cabinets for 12 h at night and returned to the glasshouse after each night. The night temperatures were set at 20 (control), 5, 2 and -1°C ± 1°C for 6 nights, with day temperatures in the glasshouse maintained at 30°C ± 1°C. Relative electrical conductivity (REC), photosynthesis and chlorophyll fluorescence were measured after the 6-day treatment. The plants were then returned to the glasshouse where reproductive partitioning and fresh and dry weights were measured 4 weeks after the treatment.

Measurements

REC test

Three 10 mm leaf discs were taken using a cork borer from two young fully expanded leaves from each plant per pot and placed in separate test tubes with 10mL de-ionised water. The test tubes were left to stand for 30 minutes before EC of the solution was measured using a calibrated EC meter. The test tubes were capped and autoclaved for 15 minutes at 121°C at a pressure of 103kPa. The EC of the solution was measured when the solution had cooled to 20°C, and REC was calculated .

Photosynthesis and chlorophyll fluorescence

Photosynthesis rates were measured using an infrared gas analyser (IRGA) portable photosynthesis system (ADC) between 1000 and 1400 h. Light intensity was set at ambient glasshouse conditions to suit the surroundings where measurements were taken, 1500 $\mu mol/m^2.s$. Carbon dioxide concentration in the chamber was fixed at 330 µL/L and the temperature and relative humidity were set to ambient conditions. Light-adapted chlorophyll fluorescence [quantum yield of photosystem II (PSII) (Fv'/Fm')] was measured using a chlorophyll fluorometer (Walz PAM-200). Measurements were taken on the youngest fully expanded leaf following the 6-night treatment at the same time as the REC and photosynthesis measurements.

Dry weights and reproductive partitioning

Plant mapping was carried out by counting the total fruiting (squares and boll) sites and retained and aborted fruit. Leaf, stem and fruit were weighed and then dried at 70^{o}C in a forced draught oven for 48 h, then weighed again. Reproductive partitioning was calculated as the ratio of reproductive dry weights (squares, flowers, green bolls) relative to the total above-ground dry weight (Bange and Milroy 2004). Fresh and dry weights of both plants and fruit were measured. Dry weight percentage was calculated by dividing the dry weights by the fresh weights. The data were analysed using analyses of variance in Genstat 8th edition.

Results and Discussion Electrolyte leakage assay using water baths

The assay temperature of 0°C for 2 h was most appropriate to differentiate cold tolerance between cultivars (Fig.1a and 1b). Temperatures higher and lower than 0°C gave closer REC values between DP16 and Namcala cultivars. Relative electrical conductivity measurements at 0°C for 2 hours are suitable and enable data to be obtained rapidly (see Fig. 1a). Similar results were also achieved at 0°C and -2°C for 4 hours (Fig. 1b). The -2 and -5°C assays for 2 h did not work well as there was insufficient time to cool the test tubes to the assay temperature, whereas at 0°C for 2 h, the melting ice in the water bath had greater capacity to absorb heat.

Whole plant chilling

A summary of the analyses of variance from the whole plant chilling experiment is presented on Table 1. Differences in REC, photosynthesis and chlorophyll fluorescence for DP 16 compared to Namcala were greatest (P<0.05) at 5°C (Fig. 2a, 2b, 2c). DP 16 had higher (P<0.05) REC at 5°C than the control, indicating greater structural damage than Namcala. Leaf photosynthetic rates for DP16 decreased (P<0.05) compared to the control whereas photosynthetic rates in Namcala remained constant. At 2°C and -1°C, photosynthetic rates decreased by 37% and 63%, respectively, for both cultivars indicating chilling stress. Plants showed evidence of photoinhibition (reduced Fv'/Fm') following the cold treatments which followed the same trend as photosynthesis. Visual differences including wilting and yellowing of leaves were also observed in the cold treatments (≤ 5°C) but not in the control. Hence, for whole plant assays, Namcala showed evidence of greater cold tolerance than DP16 after exposure to 5°C for 6 nights. There were no differences in REC, photosynthesis and chlorophyll fluorescence between cultivars at temperatures below 5°C, which could be due to structural damage in the leaf cellular membrane to both cultivars.

Both DP16 and Namcala exposed to 5°C and 2°C recovered and started producing more fruiting sites once they were returned to the glasshouse. However, DP16 plants exposed to -1°C died after 4 weeks while Namcala remained alive. DP 16 had a higher (P<0.05) dry weight percentage at -1°C (Fig. 2d) because the plants were dehydrated due to tissue damage. The probability of boll retention decreased with decreasing temperature (Fig. 2e). At 1°C for 6 nights, all DP16 plants were dead and therefore had no fruit retained (Fig. 2e). Both cultivars exposed to temperatures ≤ 5°C had lower (P<0.05) reproductive partitioning compared to the control (Fig. 3). The data from the whole plant treatment support the cold water bath assay confirming DP16 to be less cold tolerant than Namcala.

Developing methods that can rapidly screen commercial cotton cultivars for cold tolerance could potentially lead to better cultivar selection for maintaining yields in marginal, cooler

climates in Australia's cotton growing regions. An assay to distinguish cold tolerance in cotton cultivars has been successfully developed in this study using a fixed time (2 hrs) technique at 0°C. The cultivar Namcala was more cold tolerant than DP16. Future assays should be conducted on field-grown cultivars as there is a challenge in relating growth room studies to field conditions . This is particularly true for cold acclimation which is known to occur in the field . Cold acclimation is associated with several physiological, biochemical and anatomical alterations in the plant . These include increases in soluble sugars and proteins as well as modifications of membrane composition leading to better tolerance to low temperatures. Possible cold acclimation has been demonstrated in cotton production. Following day and night exposure of cold temperatures to 11-day old cotton seedlings, plants became hardened . Little is known about what triggers possible acclimation and its effect on cotton yield and development. The development of this assay provides the foundation for future research into monitoring and screening cold tolerance in cotton.

Cultivar difference in REC in response to the cold stress was found only for the 5°C treatment for 6 nights. When the plants were subjected to temperatures below 5°C, too much cellular damage occurred to both cultivars to show any difference in REC. The increase in dry weight of the crop depends on the balance between photosynthesis and respiration (Hearn and Constable 1984). Reductions in photosynthetic rates of whole plants exposed to temperatures ≤ 5°C suggest that the processes of growth are being impaired. Cotton yield is determined by number of fruiting sites, fruit retention probability and boll weight. Cotton fruits have a period of 7-10 days (from flowering) when they are highly susceptible to abortion. If the actual structural mass of the square or boll is less than a threshold fraction of its potential at a moment of susceptibility, then the square or boll is shed . This was demonstrated in our whole plant experiment, where all squares and bolls in DP16 cultivars at -1°C where shed. Cool night temperatures at approximately 16.5°C enhanced vegetative development and resulted in higher boll setting . This study has shown that cold night temperatures ≤ 5°C can have the opposite effect. Hence, these new protocols may assist in screening for cold tolerant cotton cultivars and breeding lines. Breeding can then be directed towards developing cultivars that can maintain yield and quality when exposed to low temperatures.

Conclusion

A water bath assay at 0°C for 2 h and subsequent whole plant assay at 5 °C for 6 nights developed in this study has shown that cotton cultivar Namcala is more cold tolerant than DP16. These protocols have the potential to be used for screening cold tolerance in cotton breeding lines and cultivars.

References

Captions of Figures:

Fig. 1. Relative electrical conductivity (REC) for leaf discs of glasshouse-grown cultivars (DP16 and Namcala) exposed to water bath temperatures of 25°C (control), 3°C, 0°C, -2°C, -5°C for (a) 2 h and (b) 4 h. The vertical lines represent the l.s.d. at P=0.05.

Fig. 2. Effect of cold treatment at 20°C (control), 5°C, 2°C and -1°C for 6 nights on whole plant (a) relative electrical conductivity, (b) chlorophyll fluorescence, (c) photosynthesis on the day after the cold treatment, and (d) dry weight percentage and (e) boll retention at 4 weeks after the end of the cold treatment. Vertical lines indicate l.s.d values at P = 0.05.

Fig. 3. Effect of cold treatment at 20°C (control), 5°C, 2°C and -1°C for 6 nights on reproductive partitioning. Vertical line indicates l.s.d value at P = 0.05.

Table 1. Probability of cultivar and temperature main effects and cultivar x temperature interactions for whole plant treatments at 20°C control, 5°C, 2°C and -1°C for 6 days. * - P<0.05, ** - P<0.01 and n.s. - not significantly different at P=0.05.

Variable	Cultivar	Temperature	Cultivar*Temperature
REC	*	*	**
Photosynthesis	*	*	**
Chlorophyll fluorescence	*	*	**
Boll retention (%)	*	*	*
Dry weight percentage (%)	n.s	*	*
Reproductive partitioning	n.s	*	n.s

Figure 1.

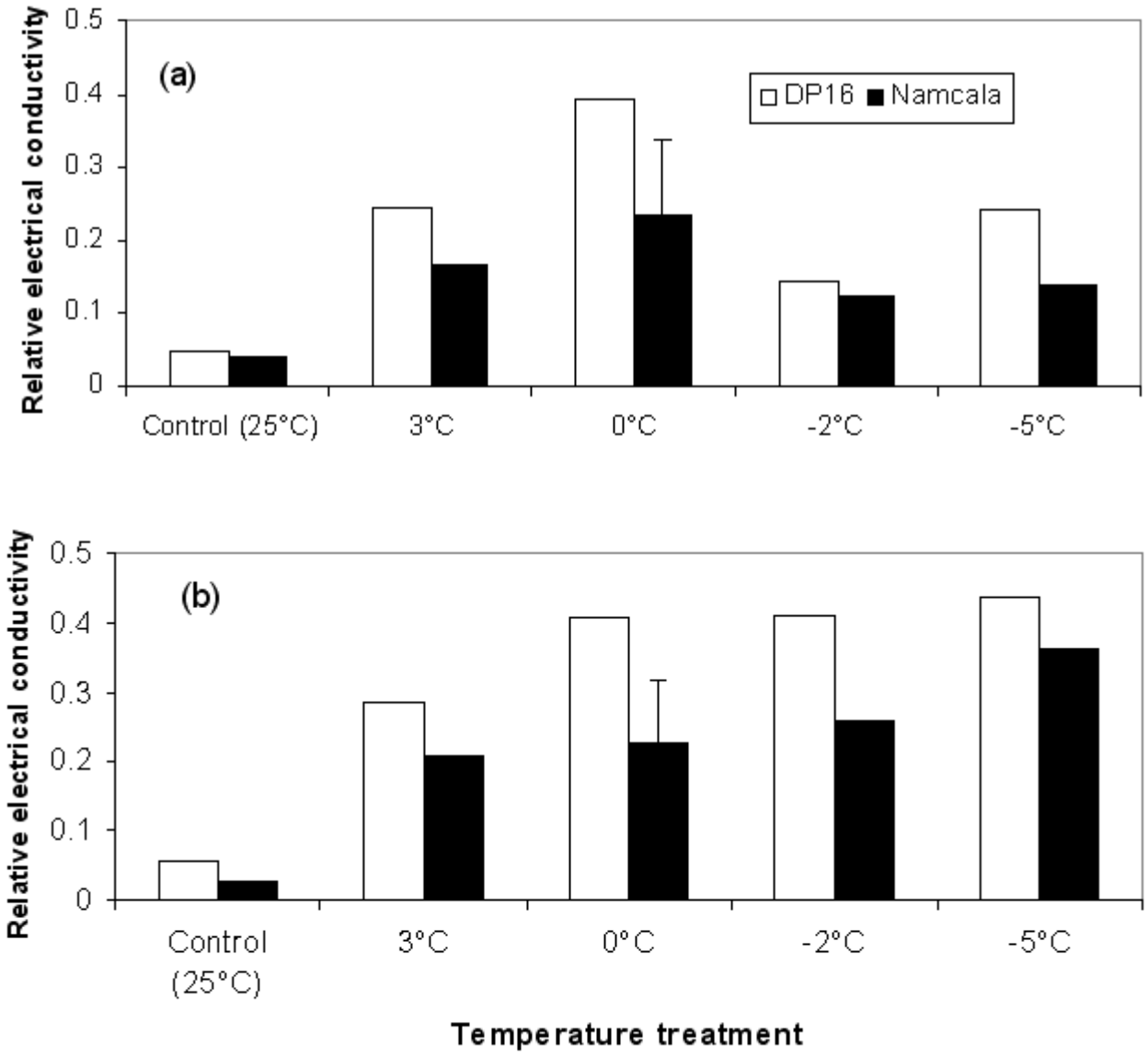
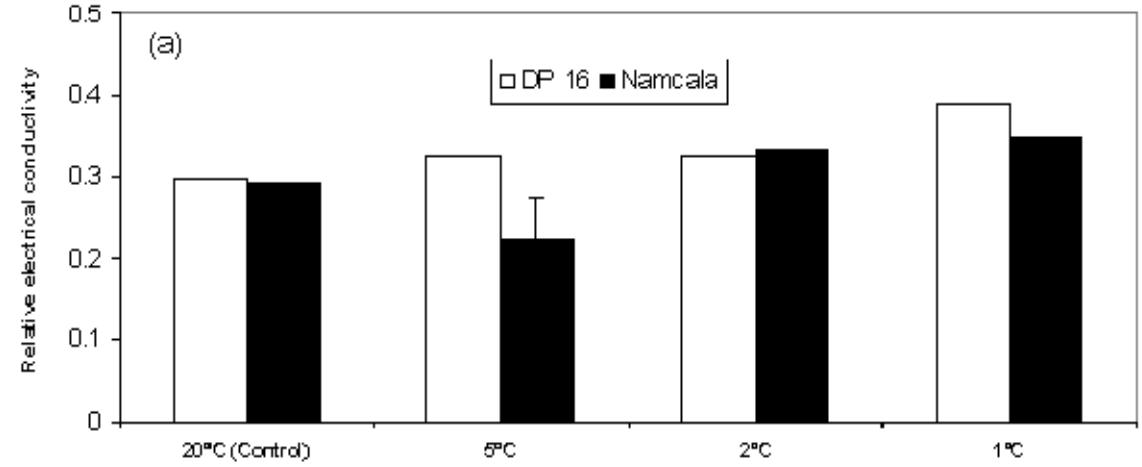
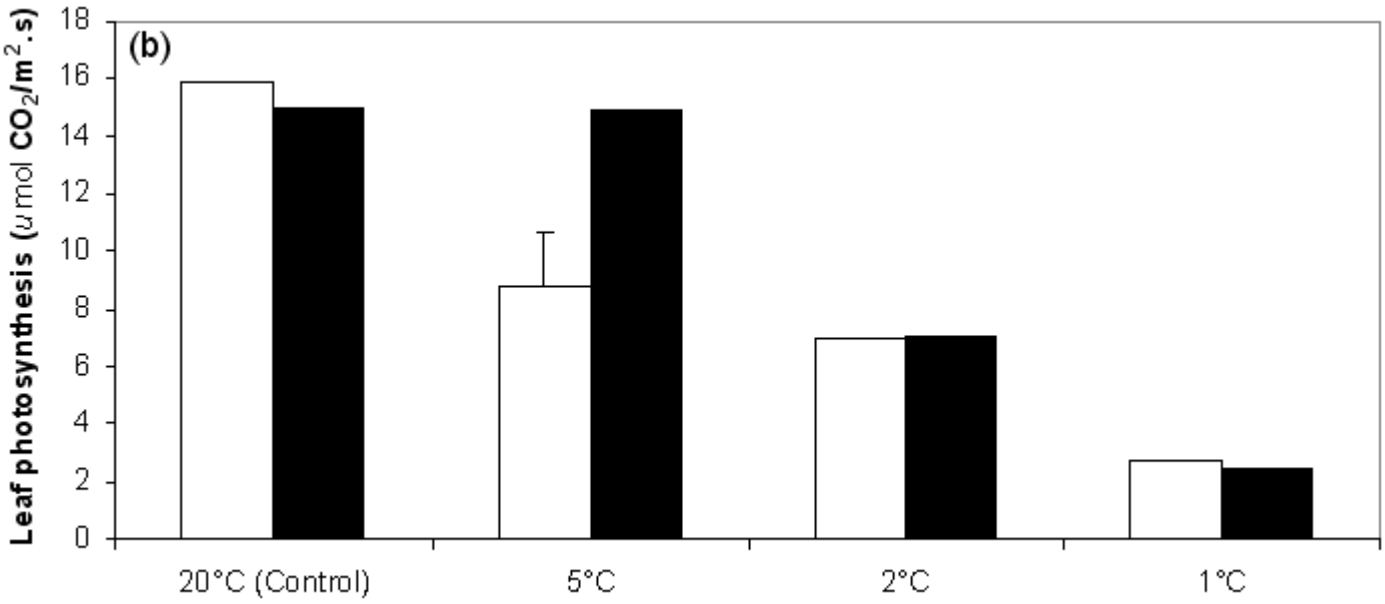
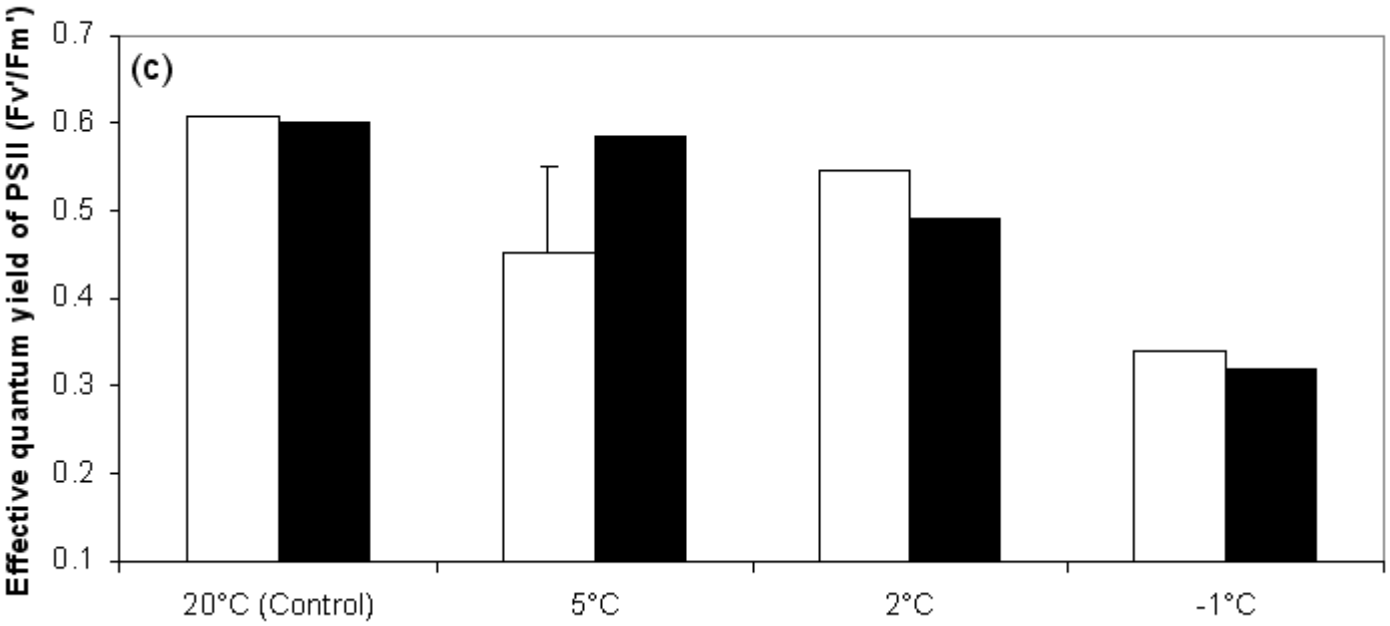
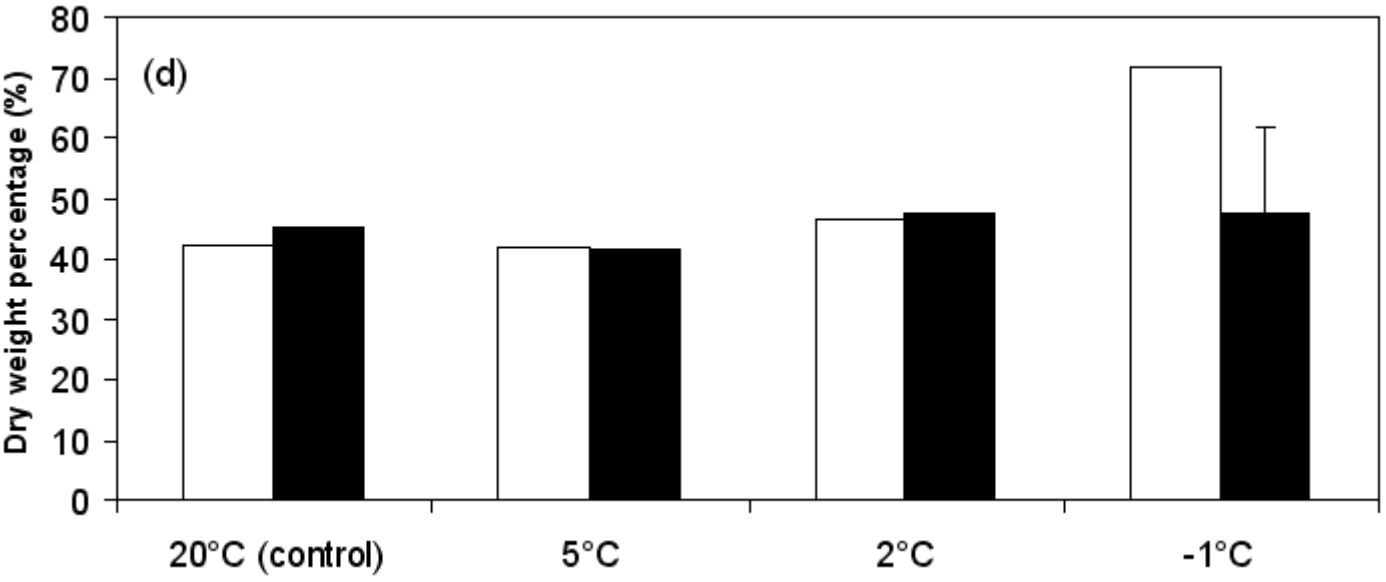

(a)
DP16
Namcala
Relative electrical conductivity
0.5
0.4
0.3
0.2
0.1
0
Control (25°C)
3°C
0°C
-2°C
-5°C
(b)
Relative electrical conductivity
0.5
0.4
0.3
0.2
0.1
0
Control (25°C)
3°C
0°C
-2°C
-5°C
Temperature treatment

Figure 2.


(a)
DP 16
Namcala
Relative electrical conductivity
0.5
0.4
0.3
0.2
0.1
0
20°C (Control)
5°C
2°C
1°C


(b)
Leaf photosynthesis (u mol CO2/m2.s)
18
16
14
12
10
8
6
4
2
0
20°C (Control)
5°C
2°C
1°C


(c)
Effective quantum yield of PSII (Fv'/Fm')
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20°C (Control)
5°C
2°C
-1°C


(d)
Dry weight percentage (%)
80
70
60
50
40
30
20
10
0
20°C (control)
5°C
2°C
-1°C

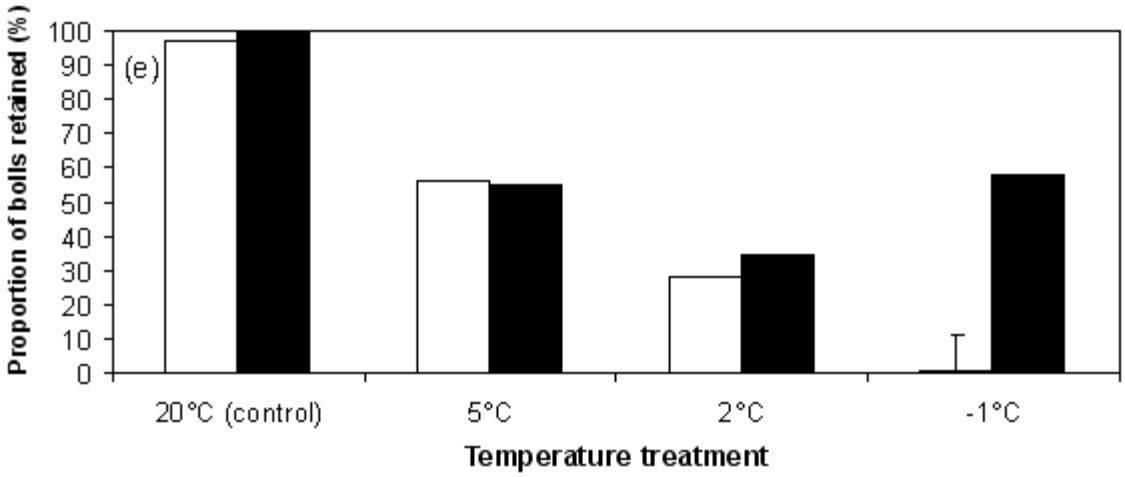
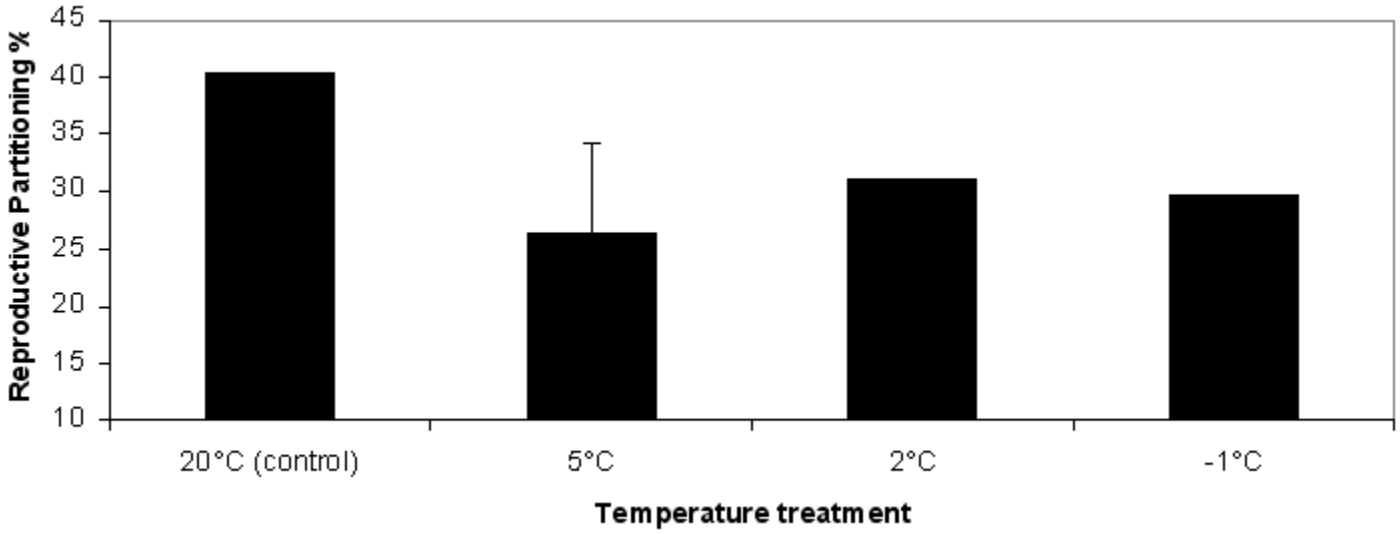

Proportion of bolls retained (%)
100
90
80
70
60
50
40
30
20
10
0
(e)
20°C (control)
5°C
2°C
-1°C
Temperature treatment

Figure 3.


Reproductive Partitioning %
45
40
35
30
25
20
15
10
20°C (control)
5°C
2°C
-1°C
Temperature treatment