Association of mid-reproductive stage canopy temperature depression with the molecular markers and grain yields of chickpea (*Cicer arietinum* L.) germplasm under terminal drought

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ARTICLE INFO

ABSTRACT

Canopy temperature depression (CTD) has been used to estimate crop yield and drought tolerance. However, when to measure CTD for the best breeding selection efficacy has seldom been addressed. The objectives of this study were to evaluate CTD as a drought response measure, identify suitable crop stage for measurement and associated molecular markers. CTD was measured using an infrared camera on 59, 62, 69, 73, 76 and 82 days after sowing (DAS) and the grain yield, shoot biomass and harvest index (%). CTD recorded at 62 DAS was positively associated with the grain yield by 40% and shoot biomass by 27% and such association diminished gradually to minimum after 76 DAS. Moreover, CTD at 62 DAS also showed similar positive association with the grain yield recorded in two previous years ($r = 0.45^{***}$, 0.42^{***}). Genome-wide and candidate gene based association analysis had revealed the presence of nine SSR, 11 DArT and three gene-based markers that varied across the six stages of observation. Two SSR markers were associated with CTD through crop phenology or grain yield while the rest were associated only with CTD for computing marker-trait associations (MTAs). The phenotypic variation explained by the markers was the highest at 62 DAS. These results confirm the importance of continued transpiration and the ability of the roots to supply stored soil water under terminal drought. The selection for grain yield through CTD is done best 15 days after the mean flowering time.

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1. Introduction

Chickpea (*Cicer arietinum* L.) is the third most important grain legume crop in the world, with a total production of 11.6 million tons from an area of 13.2 million ha and a productivity of 0.88 t ha^{-1} (FAOSTAT, 2011). Its seeds are protein-rich alternatives of animal protein in human diet. Chickpea is largely grown in arid and semi-arid environments in Asia and Africa where more than 80% of the annual rainfall is received during rainy season (June–September). The rainfall variability within the region is usually high, leading to varying intensities of drought.

Terminal drought is one of the major stresses limiting crop yield in chickpea. Genetic improvement for better drought adaptation can be a long-lasting and less-expensive solution for drought management than the agronomic options. However, understanding yield maintenance under drought becomes increasingly difficult (Tuberosa and Salvi, 2006), due to the numerous mechanisms that plants can use to maintain growth in conditions of low water supply. Therefore, breeding for drought tolerance requires knowledge of the type and intensity of drought and the various traits and mechanisms employed by the plant to overcome the drought effects. Moreover it is also important to rank and prioritize the traits/mechanisms on the basis of their strength of contribution to drought adaptation. A physiological trait-based breeding approach is increasingly sought as it increases the probability of crosses resulting in additive gene action (Reynolds and Trethowan, 2007; Wasson et al., 2012). For better success in drought tolerance breeding, the traits of choice need to be causal rather than the effect (Kashiwagi et al., 2006) and an integrator of the responses to events

across the whole life cycle e.g., transpiration efficiency, partitioning coefficient or carbon isotope discrimination (Krishnamurthy et al., 2013a,b).

Plant water balance is a direct measure of drought response but most of the related measurements such as shoot water potential, osmotic adjustment or stomatal conductance do not support a high-throughput phenotyping required for characterizing a larger population. Transpiration is the major cause of changes in leaf temperature, and there is a direct relationship between leaf temperature, transpiration rate, leaf porosity and stomatal conductance (Jackson et al., 1981; Jones et al., 2002, 2009; Rebetzke et al., 2013). As long as the plants continue to transpire through open stomata the canopy temperatures could be maintained at metabolically comfortable range—otherwise higher temperature would slow or retard the vital enzyme activities. Stomatal closures for a considerable period of time are known to increase the leaf temperature (Kashiwagi et al., 2008) and maintenance of a cool canopy during grain filling period in wheat is an important physiological response for high temperature stress tolerance (Munjal and Rana, 2003). Canopy temperature differences have been shown to correlate well with the transpiration status in rice, potatoes, wheat and sugar beet (Fukuoka, 2005).

Thermal infrared imaging, or infrared thermography (IRT), to measure the canopy or leaf temperature, are the twin approaches that measure the extent of evaporative cooling occurring in a crop canopy and allow a remote sensing of the plant water balance. Between these two approaches, thermal infrared imaging through an infrared camera offer several benefits compared with temperature sensors, most importantly the facility for spatial resolution and the ability to sample larger area. Most infrared cameras currently have arrays of 320×240 sensor elements, which mean that >75,000 individual temperature readings are recorded in a single image. This allows more precise measurements in a fraction of the time needed to perform several replicate readings per plot, which is also prone to error due to changing environmental conditions between measurements. Canopy temperature is one such integrative trait that reflects the plant water status or the resultant equilibrium between root water uptake and shoot transpiration (Jones, 2007; Berger et al., 2010). Canopy temperature has been used successfully as selection criteria in breeding for drought-prone environments (Blum et al., 1989; Fischer et al., 1998; Balota et al., 2008; Jones et al., 2009).

Deviation of temperature of plant canopies from the ambient temperature, also known as canopy temperature depression (CTD) (=air temperature (T_a) – canopy temperature (T_c)), has been recognized as an indicator of overall plant water status (Ehler, 1973; Jackson et al., 1981; Blum et al., 1982; Idso, 1982; Sivakumar, 1986; Penuelas et al., 1992; Balota et al., 2008) and facilitate in evaluation of plant response to environmental stress like tolerance to heat (Amani et al., 1996; Reynolds et al., 1998) and drought (Singh and Kanemasu, 1983; Blum et al., 1989; Rashid et al., 1999; Royo et al., 2002). CTD is positive when the canopy is cooler than the air and this value has been associated with yield increase among wheat cultivars at CIMMYT (Fischer et al., 1998) and pearl millet (Singh and Kanemasu, 1983). The thermal imagery system is a powerful tool as it can capture the temperature difference of plant canopies quite rapidly. Developmental patterns of terminal drought in peninsular India is more predictable across years as the growing season is devoid of major rains (Johansen et al., 1994) and the homogeneity of the drought stressed crop was often better than the irrigated crop (Krishnamurthy et al., 2010, 2013a,b). To test any given assumption, it is important to select a population that is elaborately characterized and well known to be diverse not only for drought but also for cross stress reactions. The mini-core collection of chickpea germplasm is assembled based on morphological and agronomic diversity (Upadhyaya and Ortiz, 2001) and also been characterized

for most biotic and abiotic stress reactions (Upadhyaya et al., 2013). A subset of extremely contrasting accessions ($n=84$) were chosen for checking the reaction in canopy temperature. Molecular markers and QTLs have helped in a rapid introgression of specific traits such as the root traits and the transpiration efficiency in chickpea and accelerated the progress of stress tolerance breeding (Varshney et al., 2013; Gaur et al., 2013). Also molecular markers and genomic regions identified for higher CTD had helped for a targeted transfer of this trait in wheat (Rebetzke et al., 2013) highlighting the importance of molecular genes in breeding programs.

Therefore the objectives of this study are (i) to assess the suitability of CTD as a trait to measure the grain yield under drought (ii) to assess the crop stage at which this relationship is close and (iii) to identify molecular markers that may explain the variation in canopy temperature.

2. Materials and methods

2.1. Assembling genotyping data

The germplasm used in this study is a subset of chickpea reference collection (Upadhyaya et al., 2008). The reference collection also includes the complete set of accessions of the minicore. The available genotyping data on this set was assembled for establishing marker trait associations. In total, 1849 marker data (35 SSRs, 1157 DArT loci, 657 SNPs and 113 gene-based SNPs) were assembled from Varshney et al. (2013) and used for association analysis.

2.2. Association analysis

Mixed linear model (MLM) with optimum compression and P3D in TASSEL 4.0 version was used for computing marker-trait associations (MTAs). Both population structure and kinship relationships and among the germplasm lines was taken into consideration to avoid false positive MTAs. MTAs were considered to be significant when $p < 0.001$.

2.3. Crop management

A subset of the minicore collection of chickpea germplasm ($n=84$), consisting of all the highly tolerant ($n=5$), several tolerant (53 out of 78), none of the moderately tolerant (0 out of 74), a few of moderately sensitive (14 out of 39) and about half of the highly sensitive (12 out of 20) accessions that were previously categorized based on their drought tolerance index (Krishnamurthy et al., 2010), were field-evaluated during the post-rainy seasons of 2008–2009, 2009–2010 and 2010–2011 on a Vertisol (fine montmorillonitic isohyperthermic typic pallustert) at ICRISAT-Patancheru (17°30′N; 78°16′E; altitude 549 m) in peninsular India. The water holding capacity of these fields in lower limit:upper limit was $0.26:0.40$ cm cm^{-1} for the 0–15 cm soil layer, and $0.30:0.47$ cm cm^{-1} for the 105–120 cm soil layer. The available soil water up to 120 cm depth of the fields used was 230, 205 and 215 mm, and the bulk density was 1.35 g cm^{-3} for the 0–15 cm soil layer and 1.42 g cm^{-3} for the 105–120 cm soil layer (El-Swaify et al., 1985). The field used was solarized using a polythene mulch during the preceding summer primarily to fully protect the crop from wilt causing fungi *Fusarium oxysporum* f. sp, among other benefits and damages (Chauhan et al., 1988).

The fields were prepared into broad bed and furrows with 1.2 m wide beds flanked by 0.3 m furrows. Surface application and incorporation of 18 kg N ha^{-1} and 20 kg P ha^{-1} as di-ammonium phosphate were carried out. The experiments were conducted in a 14×6 alpha design (84 accessions) with three replications and the plot size was 4.0 m $\times$ 4 rows. Seeds were treated with 0.5%

Benlate® (E.I. DuPont India Ltd., Gurgaon, India) + Thiram® (Sudhama Chemicals Pvt. Ltd. Gujarat, India) mixture. The seed was hand sown manually at a depth of 3–5 cm with 10 cm between plant with in rows and 30 cm between rows on 31 October 2008, 31 October 2009, and 20 November 2010. About 61 seeds were used for each 4 m row and at 12 DAS the plants were thinned maintaining a plant-to-plant spacing of 10 cm. A sprinkler irrigation, 20 mm, was applied immediately after sowing to ensure uniform emergence. Subsequently, plants were grown under rainfed condition. Intensive protection against pod borer (*Helicoverpa armigera*) was provided and the plots were kept weed free by manual weeding.

2.4. Canopy temperature

The thermal images of plant canopies were recorded using an infrared camera, IR FLEXCAM (Infrared Solutions, Inc, USA) with a sensor size of 160 × 120 pixels, sensitivity of 0.09 °C and an accuracy of ±2%. The target area of the image obtained was about 30 cm × 20 cm at one of the central row of each plot, and the images were captured from north to avoid shading of the target area (Kashiwagi et al., 2008). The software SmartView 2.1.0.10 (Fluke Thermography), was used for the image analysis and the estimation of canopy temperatures after removing the soil (background) emissions (Zaman-Allah et al., 2011a). The camera was strapped on shoulder at a height of 1.0 m and the observations were recorded between 1400 and 1530 h.

2.5. Phenology recording and final harvest

The date when 50% or more of the plants had flowered was recorded as 50% flowering time of the plot, and when 80% of the pods in a plot were dried was recorded as the time of maturity for each plot. At physiological maturity, plant aerial parts were harvested at ground level from an area of (3.6 × 1.5) 5.4 m² with care to eliminate border effects in each plot, dried to a constant weight in hot air dryers at 45 °C, and total shoot dry weights were recorded. Grain weights were recorded after threshing.

2.6. Soil moisture measurements

In all the years, neutron moisture meter access tubes were installed in four spots planted with two drought tolerant (ICC 867 and ICC 14778) and two drought sensitive accessions (ICC 6263 and ICC 8058) (Krishnamurthy et al., 2010) in an adjacent broad bed in each replication and treatment. Neutron moisture meter (Depth Moisture Gauge, Model 3332, Troxler Electronic Laboratories Inc., NC., USA) readings at soil depths of 15 cm increments up to a depth of 120 cm were made before and after each irrigation as well as matching it at about 10 day intervals. The troxler soil moisture observations were corrected with a calibration curve developed for each depth separately using the data collected gravimetrically across the season. Moisture content of the surface soil (0–15 cm) was measured only gravimetrically. The water held in each soil horizon of 15 cm depth was summed up to 1.2 m.

2.7. Statistical analysis

The replication-wise values of grain yield, yield components and CTD were used for statistical analysis for each environment using one way ANOVA. Variance components due to genotypes (σ^2_g) and error (σ^2_e) and their standard errors were determined. Broad sense heritability (h^2) was estimated as $h^2 = \sigma^2_g/(\sigma^2_g + (\sigma^2_e/r))$ where r was the number of replications. For the pooled analysis, homogeneity of variance was tested using Bartlett's test (Bartlett, 1937). Here, the year (environment) was treated as a fixed effect and the genotype (G) × environment (E) interaction as random. The variance due

to (G) (σ^2_g) and G × E interaction (σ^2_{gE}) and their standard error were determined. The significance of the fixed effect of the year was assessed using the Wald statistic that asymptotically follows a χ^2 distribution. The accessions were grouped into representative groups using the means of CTDs by a hierarchical cluster analysis (using Ward's incremental sum of squares method) for characterizing them as low or high CTD accessions.

3. Results

In all the three years, the rain received prior to the cropping season was >850 mm, well distributed and more than enough to ensure complete charging of the soil profile. Rains during cropping summed to 26 mm during 15 to 30 DAS in 2008–2009, 44 mm during 9 to 19 DAS in 2009–2010 and 12.6 mm during 19 to 22 DAS in 2010–2011 delayed the onset of drought slightly but the terminal drought stress did built up (data not shown). There was another rain (39 mm) at 75 DAS during 2009–2010, but at this stage under drought stress the early or medium maturing accessions crossed the stage of responsiveness. Overall, the minimum temperatures were higher, particularly during the critical third and fourth week of December (flowering and early-podding season for the adapted germplasm), and maximum temperatures were lower during 2009–2010 (Supplementary Fig. S1). Relatively cooler minimum temperatures and maximum temperatures at vegetative period were observed in 2010–2011. The cumulative evaporation was highest during 2008–2009 cropping season that was getting lesser in subsequent years, except the reproductive period in 2010–2011, influencing the vapor pressure deficit (VPD). VPD in 2008–2009 was high and in 2009–2010 it was moderate (Supplementary Fig. S1). When the canopy temperatures were recorded on 59, 62, 69, 73 and 76 DAS during 2010–2011, the maximum temperatures remained close to 30 °C. The minimum temperature, daily evaporation and the VPDs were to some extent similar during these days but there were notable increase in all these parameters on 82 DAS (Supplementary Table S1).

3.1. Changes in temporal soil moisture pattern

Largely, the pattern and the rate of soil moisture depletion remained the same among the three seasons but the soil moisture depletion was very rapid in 2010–2011 season in the initial two weeks as a result of low relative humidity and a marginally high VPD (Supplementary Fig. S2). However, the rain that followed at 18–22 DAS minimized the soil moisture depletion. Also this year the soil moisture at harvest was slightly high. There was a large rain at 75 DAS in 2009–2010 which raised the surface soil moisture to some extent but this returned to the usual dry condition within two weeks.

3.2. Crop phenology, grain yield and yield components

The overall trial means was 46 to 50 DAS for 50% flowering across years. The range varied from 31–66 to 35–69 DAS. Similarly, the overall trial mean for days to maturity was 91 to 97 DAS and the range varied from 79–113 to 84–118 DAS across years. Mean shoot biomass production across years ranged from 3388 to 3982 kg ha⁻¹ and the range of accessions varied approximately two times. Mean grain yield across years ranged from 1627 to 1757 kg ha⁻¹ and the range of accessions varied approximately three to four times. Mean harvest index across years ranged from 42.6 to 48.3% and the range of accessions varied from 17.6 to 63.6%. The heritability of the phenological traits and the harvest index was mostly above 0.9. The range of heritability for shoot biomass was 0.5 to 0.9 and for grain yield was 0.5 to 0.8 across years (Table 1).

Table 1
Trial means and analysis of variance of 84 accessions, a subset of the minicore collection of chickpea germplasm, for phenology, shoot biomass at maturity, grain yield and harvest index in the field experiments during post-rainy seasons of 2008–2009, 2009–2010 and 2010–2011 under drought-stressed environment.

Season	Trial mean	Range of means	S.Ed	σ^2_g (F pr.)	Heritability (h^2)
2008–09					
Days to 50% flowering	49.7	35.0–68.7	1.77	64.3 (<0.001)	0.96
Days to maturity	96.7	84.3–118.0	1.60	36.1 (<0.001)	0.92
Shoot biomass (kg ha^{-1})	3388	2620–4359	400.0	1.89 (<0.001)	0.86
Grain yield (kg ha^{-1})	1627	778–2336	212.0	3.71 (<0.001)	0.48
Harvest index (%)	48.3	20.3–63.6	2.88	16.4 (<0.001)	0.84
2009–10					
Days to 50% flowering	47.0	34.3–64.3	1.61	34.4 (<0.001)	0.92
Days to maturity	92.3	79.3–113.7	2.38	29.1 (<0.001)	0.90
Shoot biomass (kg ha^{-1})	3982	3030–5805	411.9	4.19 (<0.001)	0.52
Grain yield (kg ha^{-1})	1660	686–2381	213.2	5.47 (<0.001)	0.60
Harvest index (%)	42.6	17.6–58.4	2.29	46.4 (<0.001)	0.94
2010–11					
Days to 50% flowering	46.2	31.3–66.3	2.20	25.4 (<0.001)	0.88
Days to maturity	90.6	84.3–107.3	2.10	11.1 (<0.001)	0.77
Shoot biomass (kg ha^{-1})	3953	2487–5006	340.2	3.66 (<0.001)	0.47
Grain yield (kg ha^{-1})	1757	666–2462	186.2	10.6 (<0.001)	0.76
Harvest index (%)	44.4	19.6–58.5	2.28	36.6 (<0.001)	0.92

Table 2
Interaction of genotype with year for the grain yield and its components in the subset of the minicore collection of chickpea germplasm (n = 84) during post-rainy seasons of 2008–2009, 2009–2010 and 2010–2011 under drought-stressed environment.

	Genotype	Genotype × year
	Variance component (S.E.)	Variance component (S.E.)
Shoot biomass (kg ha^{-1})	63,840(24,838)	174,150(27,931)
Grain yield (kg ha^{-1})	94,064(16,896)	17,954(4538)
Harvest index (%)	79.98(13.67)	17.41(2.28)

A pooled analysis of three years data had shown that the accession variation for shoot biomass, grain yield and harvest index were highly significant. The genotype × year interaction component was also significant but this interaction component for the grain yield and the harvest index was five times less than the genotype component (Table 2).

3.3. The extent of variation in CTD

Maximum temperatures recorded, on the days of canopy temperature measurements (59, 62, 69, 73 and 76 DAS), were close to 30 °C. At 82 DAS, it was 32 °C (Supplementary Table S1). There was a large range of variation among the accessions for CTD, at all time of observations and the range was −4.9 at 62 DAS to −8.7 at 82 DAS. The genotypic variation among the accessions was significantly different at a probability level of <0.001. The heritability of the CTD at 76 DAS was relatively high (0.65) compared to 0.21, 0.48 and 0.49 at other DAS (Table 3). The overall distribution of accessions for their CTD was, in general, normal with a characteristic gap on the lower CTD wing (Fig. 1). As two thirds of the accessions selected in this trial (n = 58 out of 84) happened to be the drought tolerant ones,

there were lower representation in the drought sensitive or lower CTD wing of the curve.

3.4. CTD relationship with grain yield

The regressions between the CTD and grain yields were positive at all the measuring days, explaining 22, 40, 29, 21 and 9% of the grain yield variation at 59, 62, 69, 73 and 76 DAS, respectively. However, the measurement taken at 82 DAS was negative and explained a very minimal grain yield variation of 4% (Fig. 2). The closest association of CTD with grain yield was obtained with CTD measured at 62 DAS. At this stage, every 1 °C increase in CTD caused 293 kg increase in grain yield ha^{-1} (Fig. 2).

The CTD measured at 62 DAS in 2010–2011 was regressed with 2008–2009 and 2009–2010 grain yields. The regression between grain yield and CTD were also positive and significant explaining 20 and 18% of the grain yield variation in the year 2008–2009 and 2009–2010, respectively (Fig. 3). The CTD of accessions measured in a day correlated very well with the subsequent day measurements demonstrating that the CTD of the accessions are largely genetic and repeatable. The correlation coefficients (r) of CTD 59 DAS verses 62 DAS, 62 DAS verses 69 DAS, 69 DAS verses 73 DAS, 73 DAS verses 76 DAS and 76 DAS verses 82 DAS were 0.86, 0.85, 0.81, 0.81 and 0.64, respectively (Fig. 4).

3.5. CTD categorization

As the closeness in association of CTD with the next subsequent measurement was deteriorating with every delay in sampling time leading to an insignificant relationship with grain yield, and the samples measured at 62, 69 and 73 DAS only explained the grain yield significantly with good level of heritability, these three sample means were used for clustering and to have representative groups

Table 3
Mean canopy temperature depression (CTD) measured at different days after sowing (DAS) for the 84 accessions, a subset of the minicore collection of chickpea germplasm, during the post-rainy season of 2010–2011 under drought-stressed environment.

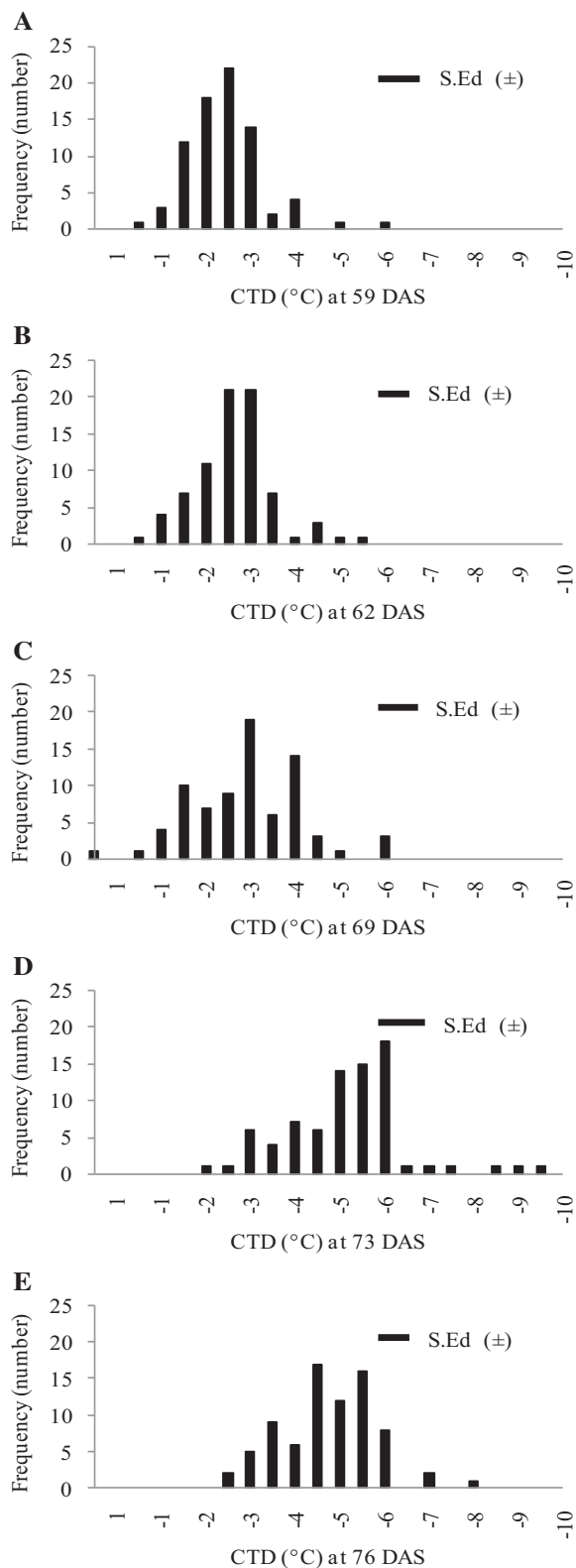| CTD at	Trial mean	Range of means	S.Ed	σ^2_g (F pr.)	Heritability (h^2)
59 DAS	−2.19	−5.68 to −0.10	0.91	1.80 (<0.001)	0.21
62 DAS	−2.38	−5.12 to −0.23	0.65	3.75 (<0.001)	0.48
69 DAS	−2.64	−5.83 to 0.53	0.87	3.73 (<0.001)	0.48
73 DAS	−4.94	−9.70 to −1.56	1.01	3.91 (<0.001)	0.49
76 DAS	−4.51	−8.46 to −1.90	0.64	6.52 (<0.001)	0.65
82 DAS	−5.08	−11.1 to −2.41	0.99	3.90 (<0.001)	0.49

Fig. 1. The distribution accessions for the canopy temperature depression (CTD) at (A) 59 (B) 62 (C) 69 (D) 73 and (E) 76 DAS during crop reproductive stage in the subset of the minicore collection (n = 84) during the post-rainy season of 2010–2011 under drought-stressed environment. The standard error of difference (S.Ed) was marked as a horizontal bar at the top right corner of each figure.

of varying CTD. This analysis yielded five groups at 85% similarity level. Based on the extent of cluster group means of CTD these can be identified as: (i) highest CTD (with CTD means at 62, 69 and 73 DAS as −1.2, −1.0 and −3.0), (ii) high CTD (−1.9, −1.8 and −4.1), (iii) moderately low CTD (−2.5, −2.8 and −5.2), (iv) low CTD (−3.1, −3.9 and −5.8), and (v) lowest CTD (−4.0, −5.2 and −8.8). The highest CTD, high CTD, moderately high CTD, low CTD and lowest CTD groups comprised of 13, 12, 42, 13 and 4 members, respectively. The extreme four groups except the moderately low CTD group is presented in Table 4. The highest CTD entries not only had the highest grain yields in all the three years but also the highest shoot biomass (Table 4). Their previous drought reactions were either highly tolerant or tolerant (Krishnamurthy et al., 2010). Similarly the high CTD group members were earlier ranked as mostly tolerant. There were 15 kabuli accessions included in this trial but none of the kabuli merited grouping in the highest or the high CTD groups.

3.6. Marker trait associations

Genotyping data generated earlier on this set (Varshney et al., 2013) coupled with phenotypic data was used for establishing marker trait associations. A total of 45 significant marker trait associations were identified for a total of 11 traits examined. For CTD trait studied at different DAS, maximum number of MTAs was observed in case of CTD at 69 DAS (10 MTAs). The p value for these MTAs ranged from 6.5×10^{-3} to 1.7×10^{-3} and phenotypic variation explained (PVE) ranged from 10.31 to 29.89%. Among 10 markers associated with this trait eight were DArT loci (cpPb-677022, cpPb-491384, cpPb-676713, cpPb-350112, cpPb-682024, cpPb-678198, cpPb-675504 and cpPb-680058) and two SSR markers (NCPGR19, TA116). However, the maximum phenotypic variation was explained for CTD at 62 DAS (Table 5). Interestingly, the MTAs for the CTD trait are located on CaLG01, CaLG04, CaLG05, CaLG06 and CaLG07 (Supplementary Table S2). Among four MTAs for CTD_62 DAS, three were SSR markers (TA113, TA116 and TA14) explaining >20% PVE and while the DArT locus associated with this trait explained 10.29% PVE. CTD measured at 82 DAS had only one significant MTA with the SNP marker Ca_TOG898271_2_002_00001_Sep08. Nevertheless, CTD measured at 59, 73 and 76 DAS had one, three and three significant MTAs, respectively.

In addition to CTD trait, 7, 5, 5, 2 and 4 significant MTAs were also found for days to 50% flowering, days to maturity, harvest index, total shoot biomass and grain yield, respectively. The phenotypic variation explained by MTAs associated with days to 50% flowering ranged from 10.30 to 62.71%, while significant MTAs for days to maturity explained 10.28 to 40.08% PVE. Interestingly, among five markers that had significant MTAs four were SNP markers (Ca1C39501, Ct6875951, Ca1C43515 and Ca1C44194) and one was a gene-based SNP marker (ASR_193_290). Further, of four markers with significant association with grain yield, three were SSR markers (TA130, TA14 and NCPGR4) and one was SNP marker (Ca1C39501).

4. Discussion

In the present study the canopy temperature was measured at six stages between 59 and 82 DAS or early pod set to the start of maturity of early duration accessions. The best linear regression between grain yield and CTD was observed with the CTD sampled at 62 DAS. This was about 15 days after 50% flowering and the early pod-filling stage of majority of the accessions. Such an association was also demonstrated to occur at anthesis, and closely after, in bread wheat grown under dryland condition (Blum et al., 1989; Royo et al., 2002; Balota et al., 2007). In wheat, while screening
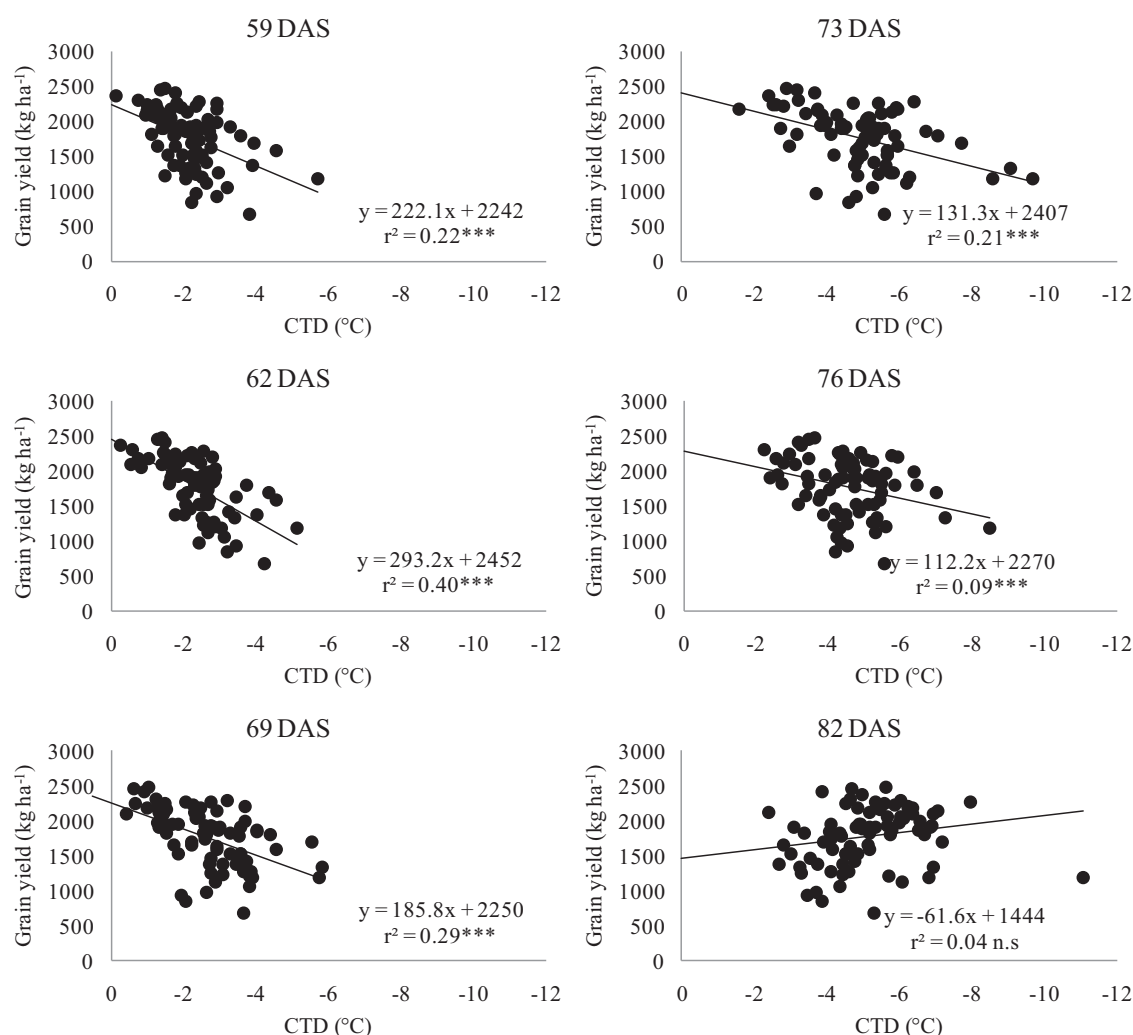
Fig. 2. The relationship between canopy temperature depression (CTD) at different days after sowing (DAS) during crop reproductive stage and the grain yield in the subset of the minicore collection ($n = 84$) during the post-rainy season of 2010–2011 under drought-stressed environment.

for heat tolerance, 10 days after anthesis was found to be the critical time for the best separation of genotypes through their CTD differences (Gowda et al., 2011). This difference in genetic discrimination stage is likely to be related to the difference in maximum leaf area development between the determinate wheat developing its maximum leaf area close to anthesis and the indeterminate chickpea at early pod fill stage or at the cessation of flowering. In addition, greater level of association of CTD with grain yield were also found to occur at 69, 73 and 76 DAS but with a diminishing level of Pearson's fit (r^2) (Fig. 2) with each delay in sampling time. This is likely due to the increasing diversification of growth stage with the delays in sampling time as some of the early duration accessions approached physiological maturity and their root system started sloughing and become less functional (Ali et al., 2002). The slope values of the CTD at 62 DAS indicated a 293 kg increase in grain yield with every 1 °C increase in CTD. However the best heritability was observed for the CTD sampled at 76 DAS. Although the ambient temperature remained close to 30 °C across the days of sampling (except at 82 DAS), every delay in sampling time increased the range of CTD from −5° to −8° reflecting the increasing build up of drought and the failure of resilience in canopy water status occurring in increasing numbers of genotypes. The ambient temperature of chickpea growing environment at the reproductive stage varies across chickpea growing regions (Silim and Saxena, 1993; Berger et al., 2010). In growing environment such as the Mediterranean

climates in Australia and Northern India the ambient temperature could be minimal (≤ 8 °C) during the reproductive duration of chickpea leading to a poor pod set and a harvest index. Under such circumstances, a warmer CTD can be desirable for a better pod set and yield advantage. On the contrary, in environments like Patancheru, India, where the ambient temperatures are known to be relatively warmer (≥ 30 °C) cooler canopy could be a desirable parameter for yield advantage. Therefore the importance of hotter or cooler canopy mainly depends on the prevalent ambient temperature and the intensity of drought. This study reveals that under drought stress in the warmer growing environments such as peninsular India a cooler canopy or a high CTD at the early pod-filling stage of crop growth is important for realizing the best drought yields in chickpea.

CTD is used as an index to determine the crop water status in many crops, as canopy temperature is heavily influenced by the air temperature compared to other environmental factors such as light intensity, wind speed and VPD (Wen-zhong et al., 2007). Dehydration avoidance is considered to be an adaptive strategy whereby plants decrease transpiration (Blum, 2009) and eventually decrease the CTD. Genotypes that are capable of regulating their stomatal activity seem to transpire less in response to high VPD under water limited conditions. This overall process makes the canopy warmer. At vegetative stage, drought tolerant genotypes had warmer canopy temperature than the sensitive genotypes in

Table 4
CTD recorded at 62, 69 and 73 days after sowing (DAS), days to 50% flowering, days to maturity, shoot biomass(kg ha^{-1}) and harvest index (%) of 2010–2011 with the grain yields recorded at 2008–2009, 2009–2010 and 2010–2011 of the highest CTD, high CTD, low CTD and lowest (inconsistent) CTD cluster group members.

Serial no.	Entries	CTD 62	CTD 69	CTD 73	Days to 50% flowering	Days to maturity	Shoot biomass (kg ha^{-1})	Harvest index (%)	Grain yield (kg ha^{-1})		
									2008–2009	2009–2010	2010–2011
Highest CTD											
1	ICC 637	−1.6	−1.3	−2.7	54	93	4307	44.0	1909	1651	1903
2	ICC 1422	−1.5	−1.5	−2.5	38	86	3865	57.7	2409	2111	2229
3	ICC 1098	−1.4	−1.0	−2.9	48	88	5006	49.2	2039	2093	2462
4	ICC 7441	−1.3	−0.6	−3.2	41	89	4445	54.8	1665	2234	2437
5	ICC 5434	−1.8	−0.6	−2.6	35	86	4422	50.4	1461	1510	2232
6	ICC 1180	−1.6	−1.5	−3.2	54	93	4998	35.9	1709	1432	1816
7	ICC 12947	−1.5	−1.3	−3.4	52	94	4398	48.0	1662	1761	2109
8	ICC 2969	−1.6	−1.5	−3.7	37	87	4145	52.1	1536	1859	2154
9	ICC 14778	−1.5	−0.9	−3.7	49	90	4738	50.9	1801	1781	2412
10	ICC 1083	−0.5	−0.4	−3.9	40	86	4031	51.9	1944	1808	2090
11	ICC 1923	−0.6	−1.2	−3.2	45	88	4475	51.1	1949	2049	2289
12	ICC 867	−0.2	0.5	−2.4	41	87	4664	51.0	1762	1933	2366
13	ICC 1164	−1.0	−1.3	−1.6	55	92	4315	50.3	1658	1631	2170
	Group mean	−1.2	−1.0	−3.0	45	89	4447	49.8	1780	1835	2205
High CTD											
1	ICC 456	−2.5	−1.5	−3.8	49	90	3789	51.3	1543	1578	1942
2	ICC 11664	−2.1	−1.8	−4.2	56	94	4178	36.4	1405	1195	1517
3	ICC 14077	−2.0	−1.7	−3.9	43	88	3644	53.3	1406	1550	1945
4	ICC 1398	−1.4	−1.4	−4.3	37	85	3699	56.6	1943	2069	2091
5	ICC 13219	−1.7	−1.3	−4.4	41	85	3884	50.3	1816	1936	1951
6	ICC 1230	−2.3	−2.4	−3.8	40	87	3979	54.8	1764	2058	2177
7	ICC 2242	−2.4	−2.6	−3.7	66	105	4312	22.4	778	1032	962
8	ICC 9586	−2.3	−2.5	−4.1	53	92	3878	46.6	1855	1544	1805
9	ICC 2065	−2.6	−1.7	−3.0	56	95	4016	40.7	1707	1356	1640
10	ICC 3325	−2.1	−2.2	−2.8	45	89	3990	55.3	1849	2066	2205
11	ICC 6279	−0.7	−1.0	−6.0	36	85	3959	55.1	1768	2015	2179
12	ICC 10399	−0.8	−1.4	−5.1	40	86	3776	54.3	1849	1802	2048
	Group mean	−1.9	−1.8	−4.1	47	90	3925	48.1	1640	1683	1872
Low CTD											
1	ICC 3218	−4.2	−3.7	−5.6	64	88	3046	22.5	1013	686	681
2	ICC 4814	−4.6	−4.5	−5.7	44	89	3741	42.1	1531	1604	1575
3	ICC 8058	−2.9	−3.8	−6.3	43	89	3093	38.5	1616	1522	1206
4	ICC 15868	−2.8	−4.0	−6.7	47	89	3732	49.8	1495	1542	1859
5	ICC 8318	−3.7	−4.4	−7.1	31	85	3426	52.1	1980	1803	1787
6	ICC 4958	−2.8	−3.7	−5.9	32	84	3747	58.5	2336	2108	2191
7	ICC 11879	−2.8	−3.8	−5.8	47	95	3686	34.5	1349	1517	1271
8	ICC 12028	−2.5	−3.6	−5.6	49	96	4335	30.4	1549	1257	1320
9	ICC 13283	−2.6	−3.6	−5.7	56	94	4760	31.8	1515	1578	1513
10	ICC 13461	−2.6	−3.6	−5.8	58	96	4414	28.8	1394	1153	1268
11	ICC 7184	−3.2	−3.7	−5.3	45	91	3918	36.2	1244	1459	1417
12	ICC 9402	−3.1	−3.8	−5.3	57	97	3999	25.9	1369	1099	1046
13	ICC 11944	−2.8	−4.0	−5.1	50	91	3987	45.3	1771	1935	1831
	Group mean	−3.1	−3.9	−5.8	48	91	3837	38.2	1551	1482	1459
Lowest CTD											
1	ICC 4872	−3.0	−3.9	−9.7	34	87	2487	47.3	1580	1946	1169
2	ICC 9002	−5.1	−5.7	−8.6	47	88	3392	49.8	1709	1928	1187
3	ICC 12155	−4.3	−5.5	−7.7	43	86	3484	48.0	1678	1638	1682
4	ICC 13863	−3.4	−5.8	−9.1	39	86	2654	50.3	1528	1651	1336
	Group mean	−4.0	−5.2	−8.8	48	87	3004	48.8	1624	1791	1344
	Environmental mean	−2.4	−2.6	−4.9	46	91	3953	44.4	1627	1660	1757

chickpea (Zaman-Allah et al., 2011a), cowpea (Belko et al., 2012) and wheat (Rebetzke et al., 2013) due to lower leaf porosity or more closed stomata. Also at this stage the ambient air temperature regimes are relatively cooler and the resultant CTD is within the comfort zone for plant metabolism. However, this pattern is not the same at reproductive stage because, increased grain yield, biomass and harvest index rely upon and were associated with reduced canopy temperature in wheat cultivars (Rebetzke et al., 2013). It is revealing that, cooler canopy temperature contributes to drought yield at reproductive stage and this phenomenon may be hard to achieve without the help of an adequately active, deep and prolific root system (Lopes and Reynolds, 2010; Rebetzke et al., 2013). However, few genotypes in this study had a good grain yield with a moderate CTD value seemingly due to their balanced transpiration.

Plot wise canopy temperature measurement using portable IR FlexCam® S seems highly advanced and reliable for screening drought tolerant genotypes in field condition in comparison to leaf based canopy temperature measurement using commercial infrared thermometers (Berger et al., 2010; Wang et al., 2013) as the thermal camera captures the whole crop canopies of many plants in a plot helping to minimize the sampling error compared to spot measurements (Kashiwagi et al., 2008). Other additional advantages are simultaneous measurement of the crop canopy area by the camera and the associated software that helps to quantify the range and mean canopy temperature and to remove the background (soil) temperature. The water requirement of a smaller canopy can be expected to be small and still resulting in a cooler canopy. This necessitates a simultaneous measurement of canopy size for validating the worth of a cool canopy. Such crop canopy area
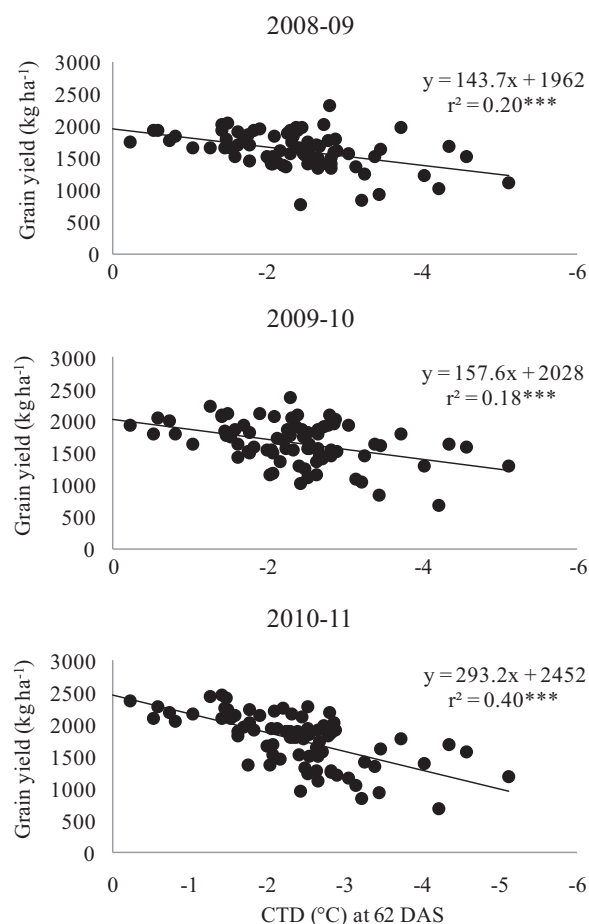
2008-09

$$y = 143.7x + 1962$$
$$r^2 = 0.20{***}$$

2009-10

$$y = 157.6x + 2028$$
$$r^2 = 0.18{***}$$

2010-11

$$y = 293.2x + 2452$$
$$r^2 = 0.40{***}$$

CTD (°C) at 62 DAS

Fig. 3. The relationship between canopy temperature depression (CTD) measured at 62 days after sowing (DAS) in 2010–2011 and the grain yield of the subset of the minicore collection ($n = 84$) during post-rainy seasons of 2008–2009, 2009–2010 and 2010–2011 under drought-stressed environment.

measurements as proportions of ground area made in this study ranged from 0.86 to 0.99 and also the incorporation of canopy area as an additional variable to explain grain yield did not improve the closeness of fit and therefore the CTD alone was considered to explain yield in this study. Additional advantage of this method is the possibility of imaging a large number of plots in a field trial in one go, allowing comparison of differences in canopy temperature among genotypes as demonstrated in rice (Jones et al., 2009).

This high throughput imaging technique is suitable for comparing genotypes in a large-scale without any error due to changing environmental conditions between measurements (Berger et al., 2010) with the limitation of increased size of the ground plot for each genotype in response to the infrared camera height (Sepulcre-Cantó et al., 2007).

In an earlier study, the whole minicore chickpea germplasm was characterized for drought reaction using a drought index that heavily depends on the grain yield performance under terminal drought (Krishnamurthy et al., 2010). Four out of five accessions that were grouped as highly drought tolerant accessions previously displayed highest CTD here confirming that their drought tolerance strategy is maintenance of an able root system for supply of enough soil water. Similarly, majority of the accessions categorized as drought tolerant previously also grouped themselves into high CTD group here while the sensitive ones as low CTD ones. Also entries like ICC 4958, the best rooting and yielding accession, displayed a low CTD due to its earliness in maturity (Table 4). Two low CTD accessions ICC 4958 and ICC 8318 flowered early and matured at 84 DAS. Massive root and leaf senescence is known to start 15 days before the maturity of the crop and therefore these accessions were already approaching the start of maturity loosing resilience in CTD. Adaptation to both drought and salinity involves some common physiological and biochemical adjustments. Large number of highest and high CTD accessions (11 out of 23) such as ICC 456, -867, -1098, -1164, -1180, -1230, -1398, -3325, -5434, -7441 and ICC 14778 were also the drought and salinity tolerant ones (Krishnamurthy et al., 2010, 2011b). Though the mechanisms of tolerance to heat are expected to vary from drought and salinity, six of these accessions, i.e. ICC 456, -1164, -3325, -5434, -7441 and ICC 14778, were also tolerant across all the three abiotic stresses.

Along with CTD, both phenological and yield component traits were included for MTA with a purpose to detect the nature of association of these markers (direct or indirect through other traits) with CTD. Significant MTAs ($n = 45$) were established in this work. It is well established through earlier works that flowering time and yield potential of the genotypes influence the grain yields under drought (Krishnamurthy et al., 2010). Similarly CTD in this study was also established to be closely associated with the grain yields under drought. Therefore the MTA of CTD could also be due to direct effect of flowering time or the grain yield. CTD is explained by more number of markers that were located in many different linkage groups, indicating that it was controlled by many genes. Also the Gaussian distribution of the CTD means (Fig. 1), in close pattern to the grain yield, supported the polygenic control of CTD as observed in wheat (Rebetzke et al., 2013). In this study, only two

Table 5

Significant marker traits association (MTA) for canopy temperature depression (CTD) recorded at 59, 62, 69, 73, 76 and 82 days after sowing (DAS), days to 50% flowering, days to maturity, shoot biomass (kg ha^{-1}), grain yield (kg ha^{-1}) and harvest index (%) during the post-rainy season of 2010–11 under drought-stressed environment.

| Traits	Number of MTAs	Name of the marker associated with trait	P-value	Phenotypic variation explained (%)
CTD_59DAS	1	CaSTMS21	4.2×10^{-3}	10.3
CTD at 62DAS	4	TA113, TA116, TA14, cpPb-677022	6.5×10^{-3}–1.7×10^{-3}	10.31–29.89
CTD at 69DAS	10	cpPb-677022, cpPb-491384, cpPb-676713, cpPb-350112, cpPb-682024, cpPb-678198, cpPb-675504, NCPGR19, TA116, cpPb-680058	7.7×10^{-3}–1.6×10^{-4}	11.66–22.18
CTD at 73DAS	3	AGL111, NCPGR19, TA130	7.4×10^{-3}–2.1×10^{-3}	10.76–18.47
CTD at 76DAS	3	cpPb-677677, cpPb-490406, TA113	3.2×10^{-3}–1.3×10^{-3}	11.23–25.13
CTD at 82DAS	1	Ca_TOG898271_2_002_00001_Sep08	4.2×10^{-3}	11.04
Days to 50% flowering	7	TAA58, Ca1C39501, TA14, cpPb-680739, cpPb-678696, cpPb-489416, cpPb-171342	7.96×10^{-18}–1.1×10^{-3}	10.30–62.71
Days to maturity	5	TA14, ASR_193_290, cpPb-675258, TR43, TA142	9.4×10^{-3}–4.6×10^{-3}	10.28–40.08
Shoot biomass (kg ha^{-1})	2	TA27, cpPb-678284	5.2×10^{-4}–9.8×10^{-3}	9.07–33.15
Grain yield (kg ha^{-1})	4	TA130, Ca1C39501, TA14, NCPGR4	8.2×10^{-4}–2.9×10^{-3}	14.72–42.31
Harvest index (%)	5	Ca1C39501, ASR_193_290, Ct6875951, Ca1C43515, Ca1C44194	9.9×10^{-3}–1.4×10^{-3}	9.48–13.77
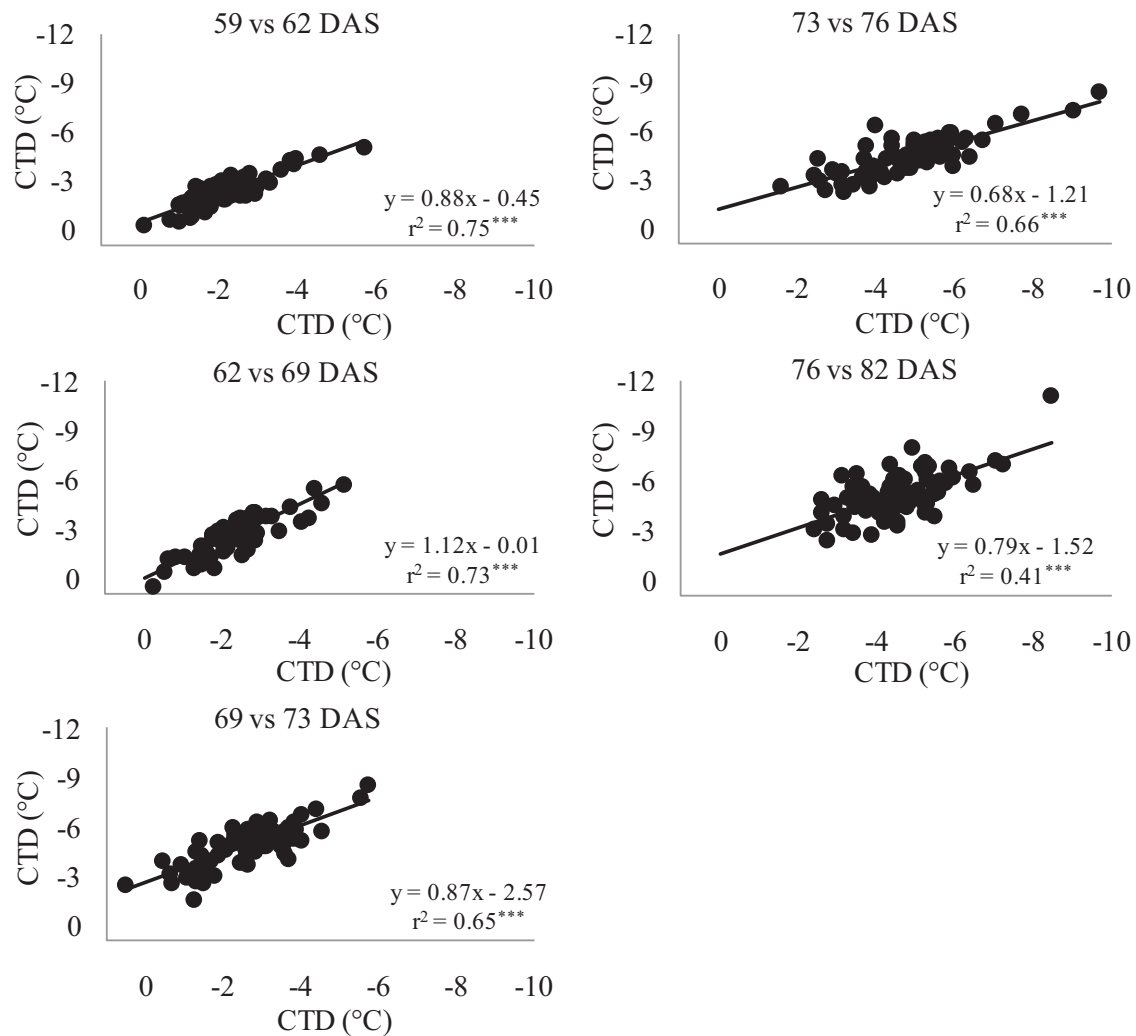
Fig. 4. The relationship of canopy temperature depression (CTD) recorded between two subsequent days of observation during crop reproductive stage in the subset of the minicore collection ($n = 84$) during the post-rainy season of 2010–2011 under drought-stressed environment. This is to show that the genotypes displayed considerable level of similarity across stages of observation.

markers were associated with multiple traits. For example, TA14 (LG6) associated with CTD at 62 DAS, was also associated with days to 50% flowering, days to maturity and grain yield. Similarly TA130 (LG4) associated with CTD at 73 DAS was also associated with grain yield. Therefore, these markers associated with more than one trait, are most likely due to pleiotropic effect of the same gene(s) (Diab et al., 2008). Except TA 14 and TA130, the remaining markers were unique in association with CTDs at various stages. However, there were almost no common markers that continue to exhibit their association across all stages of pod filling. CTD is the end result of many different direct plant processes such as root structure and function, leaf area, leaf porosity, stomatal frequency, stomatal conductance, senescence and sink strength and the importance of their contribution changing with the stage of the plant. Therefore these markers are still expected to be indirect in explaining the CTD through other traits. CTD recorded at 69 DAS exhibited MTAs with highest probability and the CTD recorded at 76 DAS resulted in the best heritability value giving high level of direct relevance to the 13 markers that were associated with CTD in these two stages. CTD is a consistent and reliable trait, which is highly linked to water use efficiency and yield potential through stomatal conductance, leaf porosity and indirectly reflects the instantaneous transpiration at the whole crop level (Reynolds et al., 1994; Fischer et al., 1998; Condon et al., 1990, 2007; Rebetzke et al., 2013). It

was also found to explain a significant proportion of yield variation under heat stress (Bennett et al., 2012). Therefore, markers specific for CTD trait seems to have a greater advantage to screen for drought response of genotypes. However, it is still necessary to validate the robustness of these markers for their association with CTD.

5. Conclusions

CTD is a stand-alone trait that measures the plant water status under a constantly changing soil-plant-atmosphere continuum. CTD measured at the mid reproductive stage explained a major proportion of the grain yield variation under terminal drought proving its worth as a proxy for grain yield. This association tended to become sparse with further delays in CTD measurement. A cooler canopy temperature at mid reproductive stage can be used as selection criterion as it ensured greater grain yield under drought. There were large number of molecular markers that explained a major proportion of the phenotypic variation in canopy temperature depression, two of them through crop phenology and grain yield. More work is required to validate the markers identified and to ascertain the pathway of marker association with CTD.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fcr.2015.01.007.

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