

Variation in carbon isotope discrimination and its relationship with harvest index in the reference collection of chickpea germplasm

Lakshmanan Krishnamurthy^{A,F}, Junichi Kashiwagi^B, Satoshi Tobita^C, Osamu Ito^D, Hari D. Upadhyaya^A, Cholenahalli L. L. Gowda^A, Pooran M. Gaur^A, Madavalam S. Sheshshayee^E, Sube Singh^A, Vincent Vadez^A and Rajeev K. Varshney^A

^AInternational Crops Research Institute for the Semi-arid Tropics, Patancheru 502 324, Andhra Pradesh, India.

^BGraduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan.

^CJapan International Research Center for Agricultural Sciences, Ohwashi, Tsukuba 305-8686, Japan.

^DUnited Nations University, 1-1-1 Minato Mirai, Nishi-ku, Yokohama 220-8502, Japan.

^EUniversity of Agricultural Sciences, GKVK Campus, Bangalore 560 065, India.

^FCorresponding author. Email: l.krishnamurthy@cgiar.org

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Abstract. Terminal drought is a major constraint to chickpea productivity. Carbon isotope discrimination ($\Delta^{13}\text{C}$), an integrator of plant behaviour influencing transpiration efficiency (TE), is an important component of yield under drought. The variation in $\Delta^{13}\text{C}$ and its association with yield was assessed in the reference collection of chickpea germplasm. Drought stress reduced shoot biomass by 36–39% and grain yield by 23%. Mean $\Delta^{13}\text{C}$ was low and the range of genetic variation was high under drought stress. Largely, high $\Delta^{13}\text{C}$ accessions were early in flowering (40–50 days), moderate in shoot biomass, high in seed yields and high in harvest index (HI). $\Delta^{13}\text{C}$ was positively correlated with seed yield in both the years under drought stress, only in 2008–09 under optimal irrigation. This positive association was very close with HI. Among the yield components, $\Delta^{13}\text{C}$ was closely associated with pod numbers per unit area and seed size under drought stress. Path coefficients showed no direct association of $\Delta^{13}\text{C}$ with grain yield but an indirect negative association through shoot biomass at maturity and a close positive association through HI. The closest association of HI or shoot biomass was seen in the maturity group of accessions that experienced the optimum terminal drought stress.

Additional keywords: genetic variability, heritability, stomatal conductance.

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Introduction

Globally, chickpea (*Cicer arietinum* L.) is the third most important grain legume crop widely grown across the Mediterranean basin, East Africa, Indian subcontinent, Americas and Australia. Approximately 90% of world's chickpea is grown rain-fed (Kumar and Abbo 2001) where terminal drought is one of the major constraints to productivity. The global demand for chickpea is projected to be 18.3 million tons in 2050 compared with a supply of 9.4 million tons in 2010, and the low income food deficit countries are expected to suffer the widest supply-demand gap (IMPACT model developed by IFPRI, Rosegrant *et al.* 2008). A large part of this deficit needs to be met through breeding for drought tolerance and yield stability. Trait-based breeding is increasingly emphasised over yield-based breeding as grain yields are heavily influenced by high genotype \times environment interactions and exhibit low heritability (Ludlow and Muchow

1990). Such trait-based breeding approaches are particularly needed to improve crop productivity under water-limited conditions for which an understanding of characteristics that confer yield advantages is necessary. Enhancing growth rates and productivity under water-limited conditions is possible only when the most relevant drought tolerance alleles are introgressed into a single genetic background.

For water-limited environments, a water-based analytical framework has been used to segregate grain yield into several components, i.e. a better water mining ability associated with the root system, superior transpiration efficiency (TE) for biomass production and a good partitioning ability for increased realisation of economic yield (Passioura 1977). The availability of large genetic variability for some of the root related traits (Kashiwagi *et al.* 2005) and the identification of robust quantitative trait loci (QTL) associated with these traits (Varshney *et al.* 2009) are

considered as major accomplishments towards breeding for drought tolerance and encourages searches for variations in other traits related to drought tolerance. Next in importance is understanding of TE and its extent of variation in chickpea. Higher plant biomass productivity per unit water absorption is important for crop improvement under water-limited conditions. However, measuring TE through gravimetric means is considered cumbersome and expensive. Although, alternate protocols are available for direct TE measurement, carbon isotope discrimination ($\Delta^{13}\text{C}$) is used as a surrogate for TE as it allows the storage of test tissue and limits the tissue requirement to a small sample. Plants are known to vary in their discrimination against heavy isotopes of carbon during photosynthesis under low intercellular CO_2 concentration, leading to a higher ^{13}C concentration in low transpiration efficient genotypes (Farquhar *et al.* 1989). Relatively early stomatal closure is thus shown to prevent further water loss and improve TE. It is well established that $\Delta^{13}\text{C}$ is a good surrogate for WUE (Sheshshayee *et al.* 2003) and the selection through the surrogate allows handling larger populations and longer sample storage. In several legumes such as bean (Wright and Redden 1995), cowpea (Ismail *et al.* 1994), groundnut (Wright *et al.* 1994), soybean (White *et al.* 1995; Tobita *et al.* 2007) and chickpea (Kashiwagi *et al.* 2006), $\Delta^{13}\text{C}$ was found to be correlated with TE. But the lack of such relationship between $\Delta^{13}\text{C}$ and TE was also shown in three legume species grown well watered (Turner *et al.* 2007), indicating that there are specific weather and soil moisture conditions where the association of $\Delta^{13}\text{C}$ becomes apparent. Recent studies indicated that there can be direct as well as indirect effects of $\Delta^{13}\text{C}$ on yield performance, and special attention is required to understand such effects (Khazaie *et al.* 2011; Mohankumar *et al.* 2011).

A reference collection of chickpea germplasm based on molecular diversity data of global composite collection (3000 accessions at 50 microsatellite loci) has been developed at ICRISAT (Upadhyaya *et al.* 2008) and documented as possessing variations to many biotic and abiotic stresses. Therefore, the objectives of this work were to (1) identify the extent of variation available for $\Delta^{13}\text{C}$ and the extreme contrasting sources from the reference collection both under terminal drought stress and optimally irrigated conditions, and (2) determine the type of relationship of $\Delta^{13}\text{C}$ with seed yield and its components.

Materials and methods

Crop management

The cultivated accessions of the chickpea (*Cicer arietinum* L.) reference collection ($n=280$) were field-evaluated during the post-rainy seasons of 2008–09 and 2009–10 on a solarised Vertisol (Krishnamurthy *et al.* 2010) (fine montmorillonitic isohyperthermic typic pallustert) at ICRISAT-Patancheru (17°30'N, 78°16'E, altitude 549 m) in peninsular India. The soil depth of the fields used in 2008–09 was ≥ 1.2 m and in 2009–10 it was 1.1 m. These soils retained ~230 mm (2008–09) and 205 mm (2009–10) of plant available water in the 120-cm (maximum rooting depth) soil profile. Later, the field was kept fallow and glyphosate 41% SL (Roundup; Monsanto

India Limited, Mumbai, India) herbicide was applied before land preparation only during 2008–09.

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 diammonium phosphate was conducted. The plot size was 4.0 m \times two rows in both the seasons. The experiments were conducted with two irrigation levels as main plot treatments (drought stressed (DS), non-irrigated except for a post-sowing irrigation; and irrigated (IR), optimally irrigated depending on the need) in a 14 \times 20 α design (280 accessions) with three replications. Seeds were treated with 0.5% Benlate (E.I. DuPont India Ltd., Gurgaon, India) + Thiram (Sudhama Chemicals Pvt. Ltd. Gujarat, India) mixture in all the two seasons. Plants for both the experiments were hand planted on 31 October 2008 and 31 October 2009 in rows 30 cm apart with 10 cm between plants at 3–5 cm depth with two seeds per hill, which were later thinned to one. During all the seasons, the fields were inoculated with *Rhizobium* strain IC 59 using the liquid inoculation method. Through perforated pipes, 50 mm irrigation was applied the next day to ensure complete emergence. Successive irrigations were applied through furrow irrigation to the irrigated treatments. The plots were well protected against pod borer (*Helicoverpa armigera*) and weeds.

Leaf sampling for carbon isotope measurements

At 63 days after sowing, 10 new fully expanded leaves from major primary branches of different plants were collected, dried below 80°C in a convection oven for 3 days and were fine-powdered in a vibrating mill. Duplicated subsamples were applied to a dynamic flash combustion type element analyser (EA-1112, Carlo Erba, Milan, Italy). An adequate quantity of CO_2 gas separated and purified by the EA was introduced to an isotope ratio mass spectrometer (Delta XP Plus, Thermo Finnigan, Hamburg, Germany) to estimate the ratio of the isotopic composition, i.e. $\delta^{13}\text{C}$ ($^{13}\text{CO}_2/^{12}\text{CO}_2$). $\Delta^{13}\text{C}$ was calculated considering $\delta^{13}\text{C}$ of the air ($\delta^{13}\text{C}$ Ca) fixed at -8‰ , and converted to a value of $+20\text{‰}$ in C_3 plants using the equation $\Delta (\text{‰}) = (\delta^{13}\text{C}_{\text{Ca}} - \delta^{13}\text{C}) / (1 + \delta^{13}\text{C} / 1000)$.

Crop phenology measurements

The date when 50% or more of the plants in a plot flowered was recorded as 50% flowering time of the plot, and when 80% of the pods were dried it was recorded as the time of maturity for each plot.

Soil moisture measurements

Neutron moisture meter access tubes were installed in four spots planted with two drought tolerant (ICC 867 and ICC 14778) and two drought sensitive (ICC 6263 and ICC 8058) accessions (Krishnamurthy *et al.* 2010) in adjacent broad-beds in each replication and treatment during both years. 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 ~10 day intervals. The Troxler soil moisture observations were corrected using 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 and presented.

Final harvest

At maturity, plant aerial parts were harvested from an area of 2.7 m² in 2008–09 and 2009–10 in each plot, dried to constant weight in hot air dryers at 45°C, and total shoot dry weights were recorded. The plants harvested at this stage were free of most pinnules due to regular leaf drop with an estimated 21% loss of plant biomass. Grain weights were recorded after threshing. HI (%) was calculated as $100 \times (\text{seed yield}/\text{total shoot biomass at maturity})$ without adjusting for the leaf drop. These HI values are slight overestimates but proportionately so across all accessions permitting comparisons. Plants from 0.5 × 0.75 m area were used for the estimation of pod number and seed number per m² and their weights. One hundred seed weight was estimated from these seed weights and numbers.

Statistical analysis

The replication-wise values of yield and yield components were used for statistical analysis of each environment using ReML considering genotypes as random. Variance components due to genotypes (σ_g^2) and error (σ_e^2) and their standard errors were determined. Environment-wise best linear unbiased predictors (BLUPs) for the accessions and others were calculated. Broad sense heritability (h^2) was estimated as $h^2 = \sigma_g^2 / (\sigma_g^2 + (\sigma_e^2/r))$, where r was the number of replications. The significance of σ_g^2 was tested against its s.e.

For the pooled analysis, homogeneity of variance was tested using Bartlett's test of homogeneity (Bartlett 1937). In pooled analysis environment was considered as a fixed effect and the genotypes as random. The variance due to genotype (G) (σ_g^2) and genotype × environment (G × E) interaction (σ_{gE}^2) and their s.e. 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 means derived were used for the correlations and the path coefficient analysis. Path analysis in this case was sought to separate the correlations into different components of direct and indirect effects due to a large number of independent variables that are expected to contribute to the dependant variable, the grain yield.

Results

Weather during crop growth season

The rains received before the cropping season (842 mm in 2008–09 and 902 mm 2009–10) was higher than the normal and ensured complete charging of the soil profile. The weather during the growing seasons varied from the long-term in time of cessation of seasonal rains. During 2008–09, between 15 and 30 days after sowing, there were rains ranging from 10 to 15 mm week⁻¹ totalling 26 mm. Such early rains during 2009–10 were between nine and 19 days totalling 44 mm (Fig. 1). The overcast and the drizzle during these periods delayed the onset of drought. There was another rain (39 mm) at 75 days after sowing during 2009–10, but this was too late for most of the accessions under drought stress (DS) as many of

them had reached physiological maturity and were early enough to promote excessive vegetative growth and lodging in most accessions under irrigation (IR). Overall, the minimum temperatures were higher, particularly during the critical third and fourth week of December (flowering and early-podding season of the adapted germplasm), and maximum temperatures were lower during 2009–10. The daily evaporation that was ~3–4 mm, increased to 5 mm and beyond 82 days after sowing in 2008–09, and 91 days in 2009–10.

Changes in soil moisture across growing season

Generally, the pattern and the rate of soil moisture depletion did not vary between the two seasons under DS except that the 2008–09 season started with greater available soil moisture, and this difference between the years was maintained till 30 days after sowing (Fig. 2). Once again, the available water in the soil profile was the lowest in 2009–10 under DS between 60 and 70 days after sowing. At complete maturity under DS the available soil water left unutilised was about the same in both years (≤ 50 mm). Most of this water was found below the 60 cm soil profile. Irrigations at the early stages of the season raised the total available soil water to initial levels, whereas as the season advanced irrigations did not fully charge the profile. It is likely that there was a poor rate of percolation of water in Vertisols with irrigations compared with slow rains, and it was found that only the top 60 cm soil got wet when irrigation was turned off at the beginning of runoff. However, this surface wetting was sufficient to fully support plant growth in the irrigated treatment (Fig. 2).

Effect of terminal drought on phenology of chickpea germplasm

The overall means for each irrigation treatment across years had shown that drought stress advanced the days to 50% flowering by 3–4 days and the days to maturity by 17 days (Table 1). The range of predicted means for days to 50% flowering did not show this effect like the means. Drought stress usually advanced flowering time. By the time early maturing accessions started flowering, the drought stress was not intense enough to be significant, and therefore the early maturing accessions flowered at the same time under both irrigation levels. On the contrary, in the very late accessions, an intense drought caused the DS plants to desiccate and dry without flowering. The accessions tested ranged and varied widely with a maximum difference of ~32 days for days to 50% flowering and ~36 days for days to maturity under DS. But these differences for days to maturity were markedly narrow under IR; 17 days in 2008–09 and 8 days in 2009–10.

Effect of terminal drought on yield and yield components of chickpea germplasm

Mean shoot biomass produced in 2009–10 was marginally more than in 2008–09 in both the moisture environments. Drought stress reduced shoot biomass by 36% in 2008–09 and 39% in 2009–10 (Table 1). The accessions varied 2-fold in shoot biomass in both the soil moisture environments and years. Pooled analysis of shoot biomass, under IR across years showed year, genotype and the genotype × year effects to be significantly different and the genotypic effect was about four times higher than the

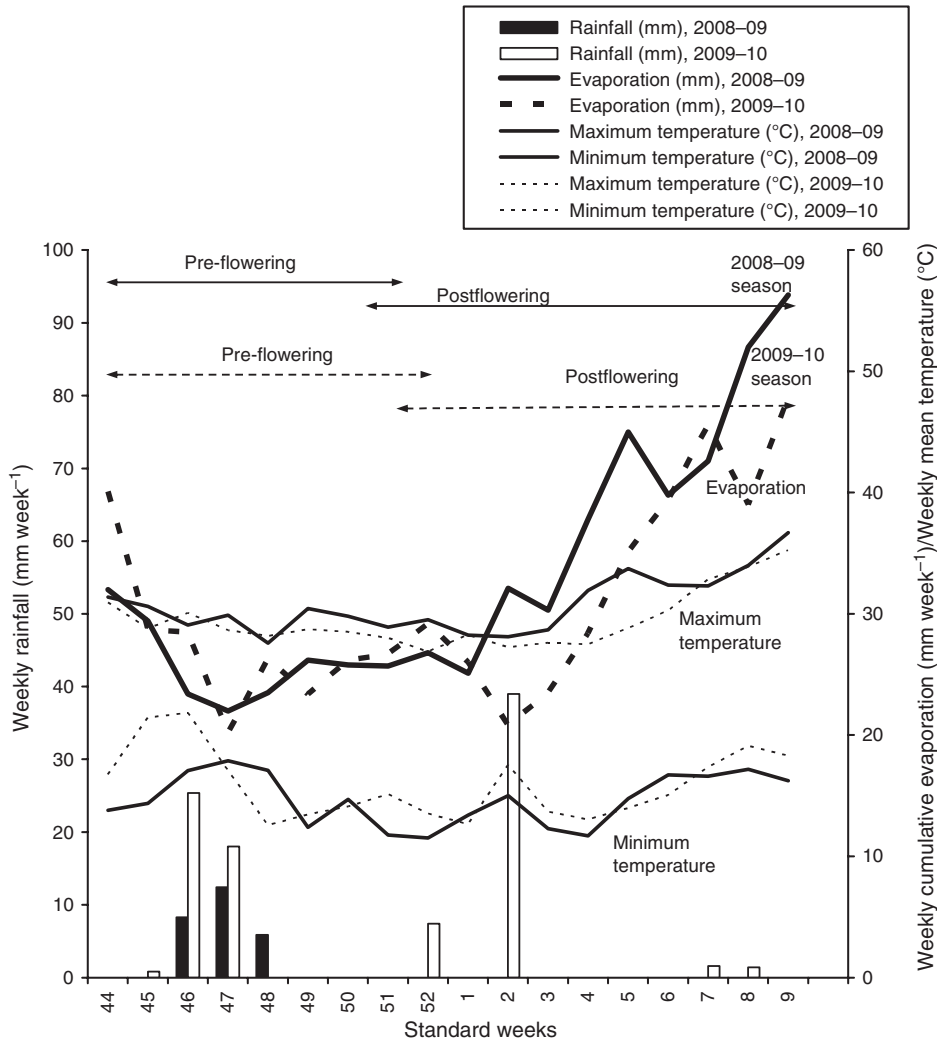


Fig. 1. Weather during the cropping seasons of 2008–09 and 2009–10 post-rainy season at Patancheru, India.

genotype \times year effect. Under DS the genotype \times year component was non-significant and the genotype effect explained the major variation. The mean seed yield was similar in both the years in the DS environment whereas it was substantially higher in the IR treatment only in 2008–09. In 2009–10, the IR yield mean and range remained close to the DS treatment as a consequence of excessive vegetative growth resulted due to a rain following irrigation. For yield, the accessions ranged more than 4-fold variation under DS and 3-fold under IR environments. Seed yield under both irrigation levels showed highly significant year, genotype and the genotype \times year effects. Under each irrigation level the genotypes explained a four-fold greater variation compared to the genotype \times year variation. The HI in general was poor in 2009–10. Drought stress increased HI. There was a significant variation among accessions for the HI. Pod number m^{-2} performance was very close to the seed yield performance. Mean 100 seed weight was similar across years and DS improved mean 100 seed weight. The accessions tested

exhibited a large variation for seed size under both DS and IR environments.

Effect of terminal drought on $\Delta^{13}C$ of chickpea germplasm

Mean $\Delta^{13}C$ was very close between the two seasons under IR conditions (20.6‰) whereas it was 19.5‰ in 2008–09 and 18.3‰ in 2009–10 under DS. Drought stress decreased this value. The decrease was substantial during 2009–10 when the drought stress was severe at the time of sampling due to less water holding capacity of the soil (Fig. 3). The range of genotypic variation was the highest under DS during 2009–10 (3.1‰) followed by DS during 2008–09 (2.7‰). This range of $\Delta^{13}C$ was the least under IR during 2009–10 (1.2‰). Highest $\Delta^{13}C$ was found under IR 2009–10 with a narrowest range among the accessions. The lowest isotopic discrimination was found under DS in the 2009–10 season with the highest range of $\Delta^{13}C$ values. Pooled analysis of $\Delta^{13}C$ under IR showed year, genotype and the genotype \times year differing significantly with

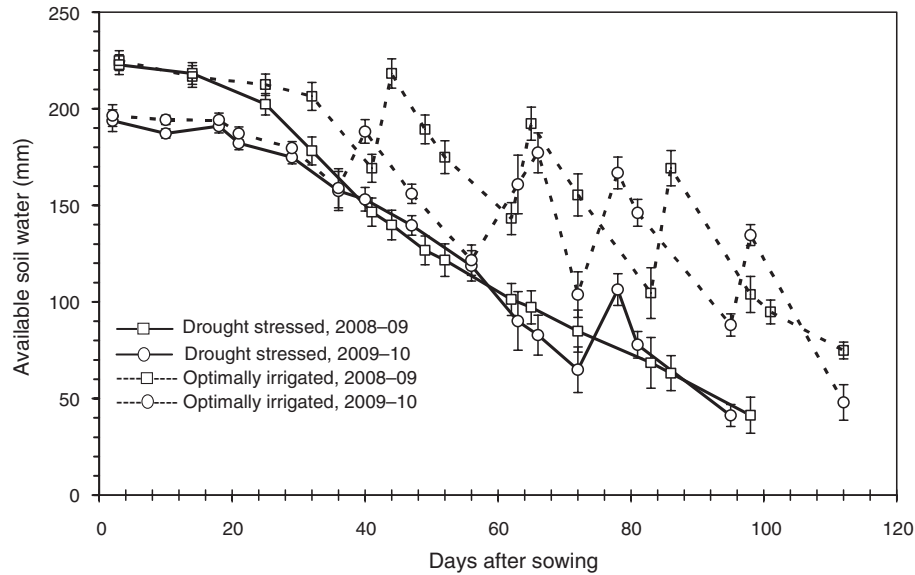


Fig. 2. Changes in available soil moisture up to a soil depth of 1.2 m across the crop growing seasons of 2008–09 and 2009–10. Vertical bars denote \pm s.e.

a large genotypic effect (0.093 ± 0.016) and comparatively smaller (0.049 ± 0.014) genotype \times year effect. Also under DS condition, all the factors were significantly different with the genotypic effect (0.268 ± 0.035) showing a far greater variance component than the genotype \times year (0.101 ± 0.022). The regressions of $\Delta^{13}\text{C}$ (as well as its rank) between the years were significant, which explained 30% variation under DS and 14% under IR.

Accession means above (high $\Delta^{13}\text{C}$) and below (low $\Delta^{13}\text{C}$) 2-folds of s.e. of difference under DS were identified and listed in Table 2. The high $\Delta^{13}\text{C}$ accessions were 20 and the low $\Delta^{13}\text{C}$ ones were 21. Most of the high $\Delta^{13}\text{C}$ accessions were the 40–50 day flowering time group with moderate shoot biomass productivity, high seed yield, high HI and small seeds.

Relationship of $\delta^{13}\text{C}$ with phenology and yield components

Correlations

$\Delta^{13}\text{C}$ was not correlated with either days to 50% flowering or maturity with the exception of the 2009–10 DS treatment (Table 3). This relationship was negative and closest in the DS crop of 2009–10 when drought stress was relatively severe. $\Delta^{13}\text{C}$ was also negatively correlated with the shoot biomass, closely in the DS treatment during 2009–10. $\Delta^{13}\text{C}$ was positively correlated at a 5% probability or closer with the seed yield under DS environment in both the years and under IR only during 2008–09. These positive correlations of $\Delta^{13}\text{C}$ were even closer with HI in these soil moisture regimes and seasons. Among the yield components, $\Delta^{13}\text{C}$ was positively associated with pod numbers per unit area in the DS environments in both the years and in year 2008–09 under IR environment. $\Delta^{13}\text{C}$ was negatively correlated with 100 seed weight only under DS in both the seasons. When similar irrigation environments of the 2 years were pooled, these correlations appeared again under DS alone with the

phenology traits and all the other yield and yield components, but under IR conditions $\Delta^{13}\text{C}$ was associated with shoot biomass, HI and pod number m^{-2} .

Path analysis

Path coefficients provide absolute measures of association, both direct and indirect, thereby assisting in assigning appropriate significance and choice for selection and use in breeding programs. The path coefficients showed that the direct association of $\Delta^{13}\text{C}$ with seed yield was close to nil in both the season and soil moisture environments, individually or pooled. Similarly the indirect association of $\Delta^{13}\text{C}$ through phenology and the yield components such as pod number m^{-2} and 100 seed weight was also close to nil. The two components through which the associations are noticed are the total shoot biomass that associated negatively, and HI that associated positively (Table 4). However, $\Delta^{13}\text{C}$ had major indirect associations with the seed yield, primarily through HI. In general, the degree of association of $\Delta^{13}\text{C}$ was more under DS than under IR through HI, followed by total shoot biomass (Table 4).

Contribution of $\Delta^{13}\text{C}$ to grain yield in groups of varying phenology

The relative association of $\Delta^{13}\text{C}$ may change across phenologically different groups of accessions that flowered from 35 to 70 days after sowing. Therefore the accessions were segregated into four groups (i.e. group 1 = accessions that took <41 days for 50% flowering ($n=5$ in DS 2008–09; $n=2$ in IR 2008–09; $n=41$ in DS 2009–10 and $n=15$ in IR 2009–10); group 2 = between 41–50 days ($n=131$ in DS 2008–09; $n=78$ in IR 2008–09; $n=111$ in DS 2009–10 and $n=66$ in IR 2009–10); group 3 = between 51–60 days ($n=118$ in DS 2008–09; $n=147$ in IR 2008–09; $n=118$ in DS 2009–10 and $n=181$ in IR 2009–10) and group 4 = accessions that took >60 days for 50% flowering ($n=15$ in DS 2008–09; $n=51$ in IR 2008–09; $n=4$ in DS

Table 1. Trial means, range of best linear unbiased predicted means of accessions (BLUPs) and analysis of variance of the 280 accessions of reference set of chickpea germplasm for phenology, shoot biomass at maturity, seed yield and a few yield components in the field experiments during post-rainy seasons of 2008–09 and 2009–10 under both drought stressed (DS) and optimally irrigated (IR) environments

Season/environment	Trial mean	Range of predicted means	s.e.	σ_g^2 (s.e.)	Heritability (h^2)
<i>2008–09, Drought stressed</i>					
$\Delta^{13}C$	19.5	17.9–20.6	0.41	0.310 (0.0367)	0.729
Days to 50% flowering	51.1	38.8–70.9	1.77	35.53 (3.12)	0.966
Days to maturity	98.2	83.2–119.7	2.08	38.78 (3.48)	0.946
Shoot biomass (kg ha ⁻¹)	3444	2259–4726	501.6	220 188 (28 546)	0.677
Seed yield (kg ha ⁻¹)	1557	510–2250	802.9	91 544 (10 202)	0.777
Harvest index (%)	45.5	11.4–60.5	3.05	80.1 (7.21)	0.943
Pods (number m ⁻²)	1358	512–2332	255.3	118 937 (12 762)	0.805
100 seed weight (g)	16.3	8.8–36.2	1.13	34.95 (2.996)	0.988
<i>2008–09, Optimally irrigated</i>					
$\Delta^{13}C$	20.6	19.6–21.3	0.38	0.211 (0.0278)	0.661
Days to 50% flowering	54.4	40.4–69.2	2.31	42.98 (3.84)	0.953
Days to maturity	115.3	109.3–125.8	1.95	7.47 (0.82)	0.816
Shoot biomass (kg ha ⁻¹)	5263	3602–6977	648.3	447 678 (62 609)	0.631
Seed yield (kg ha ⁻¹)	2057	1004–3029	311.4	138 923 (16 309)	0.737
Harvest index (%)	39.4	17.3–52.8	3.85	48.16 (4.60)	0.894
Pods (number m ⁻²)	1966	802–3297	451.7	332 582 (37 146)	0.781
100 seed weight (g)	15.7	7.2–44.6	1.14	33.97 (2.92)	0.988
<i>2009–10, Drought stressed</i>					
$\Delta^{13}C$	18.3	16.6–19.7	0.5	0.445 (0.0532)	0.722
Days to 50% flowering	48.4	34.8–65.7	2	37.78 (3.38)	0.948
Days to maturity	95.2	78.7–114.7	3.18	82.03 (7.41)	0.94
Shoot biomass (kg ha ⁻¹)	4120	2822–5499	430.9	303 086 (37 854)	0.737
Seed yield (kg ha ⁻¹)	1518	442–2314	209.3	134 255 (13 672)	0.86
Harvest index (%)	37.7	11.3–57.0	2.77	132.99 (11.60)	0.973
Pods (number m ⁻²)	1136	565–1901	197.6	75 310 (8751)	0.741
100 seed weight (g)	17.3	9.2–44.8	1.31	38.32 (3.32)	0.978
<i>2009–10, Optimally irrigated</i>					
$\Delta^{13}C$	20.6	20.0–21.2	0.29	0.095 (0.0151)	0.556
Days to 50% flowering	52.5	35.8–64.4	3.05	40.37 (3.88)	0.893
Days to maturity	112.2	108.2–116.6	1.46	3.726 (0.451)	0.723
Shoot biomass (kg ha ⁻¹)	6781	3566–8926	773.1	1 086 034 (129 241)	0.733
Seed yield (kg ha ⁻¹)	1522	694–2311	302.8	136 055 (17 861)	0.655
Harvest index (%)	22.7	10.9–37.8	3.67	38.08 (3.94)	0.828
Pods (number m ⁻²)	1299	764–2321	315.5	120 352 (18 101)	0.587
100 seed weight (g)	15.1	8.1–33.9	1.55	22.59 (2.02)	0.948

2009–10 and $n = 15$ in IR 2009–10)) and the correlations and path analysis was performed. All the correlations were significant except for the group 1 in DS and IR in 2008–09. Group 1 accessions were early and likely to have escaped drought. Phenology of group 2 was the best adapted for this region with a possible exposure to mild/moderate drought stress. Group 3 accessions were exposed to high intensities of drought whereas the group 4 accessions suffered intense drought and heat as seen through their poor range of HI and greater shoot biomass. Again in all these four groups the path coefficients showed no significant direct association of $\Delta^{13}C$ with seed yield or through any other phenology or yield component traits other than total shoot biomass and HI. Therefore, the degree of indirect associations (coefficients) is presented in Fig. 4. The salient features are as follows.

- (1) Irrespective of soil moisture, year or the phenological group, the closer association of $\Delta^{13}C$ with seed yield came through the HI.
- (2) The closest positive association of HI was seen in the 41–50 day flowering group under DS in 2009–10, but such association was closest in the >60 group when irrigated.
- (3) These associations were closer during 2009–10 when the drought stress was severe.
- (4) The indirect negative association through shoot biomass was close and tended to widen with the increase in phenology under DS as in 2009–10, whereas it improved with increasing phenology under IR. The positive association with HI was high and increased with increase in phenology under DS in 2008–09, whereas the increase was moderate under IR condition.

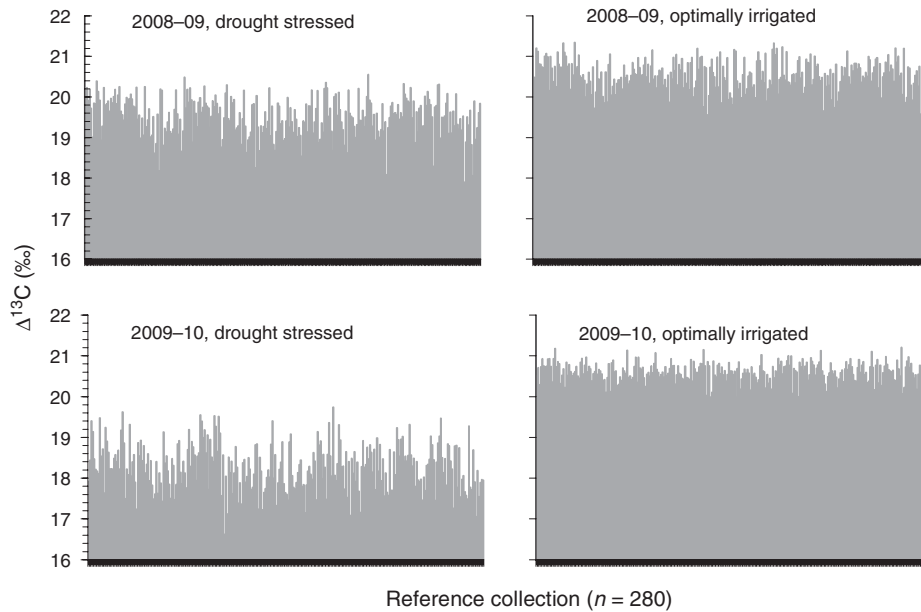


Fig. 3. Distribution of carbon isotope discrimination (‰) in the reference collection accessions of chickpea germplasm ($n = 280$) under drought stressed (DS) and optimally irrigated (IR) conditions in two (2008–09 and 2009–10) post-rainy seasons.

- (5) The relationship between $\Delta^{13}\text{C}$ and the seed yield was close and consistent in the two medium phenology groups of accessions. In the early phenology group (as these accessions probably escaped drought) the genotypic discrimination was low or inconsistent.

There was a close negative relationship between the path coefficients of shoot biomass and the HI (Fig. 5). This association was seen over all groups, but among the second and the third groups of genotypes it was very close and explained 78% of the total variation. This relationship indicated a matching trade-off between the total shoot biomass and the partitioning, and negative linkages between the two traits, i.e. shoot biomass and HI.

Discussion

Variation and possible use of $\Delta^{13}\text{C}$

The range of $\Delta^{13}\text{C}$ observed in this study compares well with earlier studies (Khan *et al.* 2004; Kashiwagi *et al.* 2006) and $\Delta^{13}\text{C}$ is seen to associate indirectly with chickpea yields particularly under drought stress. This association through plant biomass and the sink strength (HI) is expected as it explains the pathway of C accumulation. Twenty accessions of the reference collection ($n = 280$) were identified to possess the highest $\Delta^{13}\text{C}$ in this study. We find it encouraging to note that 12 of them ranked as the top drought tolerant accessions and the rest as tolerant (based on their stress yields), in a previous study that dealt with the minicore chickpea germplasm that shares 211 accessions with the reference collection (Krishnamurthy *et al.* 2010). Reports from a large number of studies involving collections of bread wheat, durum wheat and barley grown in rain-fed and irrigated environments

in Australia and elsewhere, show a positive or neutral relationship between grain yield and $\Delta^{13}\text{C}$ (as reviewed by Condon *et al.* 2004) confirming that $\Delta^{13}\text{C}$ can reflect in yield. Pod $\delta^{13}\text{C}$ in chickpea is shown to reflect water stress effects better than the leaves (Behboudian *et al.* 2000). Mature grain $\Delta^{13}\text{C}$ is expected to integrate the discrimination throughout the growth period and several reports on durum wheat had highlighted the usefulness of grain $\Delta^{13}\text{C}$ for durum wheat selection under Mediterranean conditions (Royo *et al.* 2008). However, this work has concentrated on the leaves so that a plant selection/rejection on the basis of $\Delta^{13}\text{C}$ or its molecular markers is possible within the same generation without losing the seeds. Also, a clear relationship of low $\Delta^{13}\text{C}$ is reported with both higher shoot and root DW in a wild barley germplasm (Robinson *et al.* 2000) as well as with yield and straw biomass of glaucousness variant isolines of cultivated barley (Febrero *et al.* 1998) indicating $\Delta^{13}\text{C}$'s association with other traits that confer yield advantages.

With occasional exceptions, the high $\Delta^{13}\text{C}$ accessions identified in this study were relatively early as reported in *Arabidopsis* (Mckay *et al.* 2003). These accessions also had a not-so-high shoot biomass, a high HI, greater pod numbers per unit area and smaller seeds. Similar associations were also found in wheat, groundnut (Farquhar and Richards 1984; Ehdai *et al.* 1991) and barley (Chen *et al.* 2012). There is a concern that indiscriminate selection for higher water use efficiency (WUE), as it equates with improved yield under stress might bring about serious negative consequences for the yield potential since high WUE is largely a function of reduced water use rather than a net improvement in plant production or biochemistry of assimilation (Blum 2005). It was felt that in selection programs it may constitute a marker for reduced water use commonly achieved via moderated growth, reduced leaf

Table 2. Mean carbon isotope ratio of high (two standard errors of difference above the grand mean) and low (two standard errors below the grand mean) ranking accessions out of 280 and their mean days to 50% flowering, days to maturity, shoot biomass (kg ha⁻¹), seed yield (kg ha⁻¹), HI (%), pods (number m⁻²), seeds pod⁻¹ and 100 seed weight (g) across two seasons under drought stressed (DS) environment

Serial number	Accession	$\Delta^{13}C$	Days to 50% flowering	Days to maturity	Shoot biomass (kg ha ⁻¹)	Seed yield (kg ha ⁻¹)	Harvest index (%)	Pod number m ⁻²	Seed pod ⁻¹	100 seed weight (g)
<i>High $\Delta^{13}C$</i>										
1	ICC 1422 ^A	20.14	40.3	86.5	3642	1992	56.3	1612	1.03	17.4
2	ICC 762 ^A	20.1	65.3	114.4	3456	950	25.7	954	1.2	11.1
3	ICC 15868 ^A	20.08	50.7	94.5	3077	1490	50.5	1289	1.4	11.1
4	ICC 5221	19.9	47	91.9	3147	1543	51.6	1461	1.15	11.3
5	ICC 5878 ^A	19.86	43.5	88	2878	1452	54.5	1335	1.18	11
6	ICC 11279	19.85	56.7	115	2537	389	10.2	758	1.13	9.8
7	ICC 10945 ^A	19.85	45.3	90.1	3465	1724	51.2	1677	1.14	13.9
8	ICC 8318 ^A	19.8	38.8	86.6	3367	1824	56.9	1396	0.99	19.3
9	ICC 11498	19.78	53.1	101.4	3750	1600	43.1	1451	1.24	12.6
10	ICC 1882 ^A	19.76	42.6	89.1	3532	1905	55.5	1669	1.01	14
11	ICC 4918	19.73	41.4	86.3	3607	1953	56.6	1480	1.02	18.7
12	ICC 1398 ^A	19.73	40.8	84.8	3503	1968	58.4	1383	1.06	17.9
13	ICC 12916	19.69	55	101.9	3864	1659	43.1	1217	1.28	12.1
14	ICC 10466	19.69	44.3	94.1	3461	1736	51.7	1874	1.28	10.8
15	ICC 283 ^A	19.69	42.2	90.4	3328	1817	57.3	1670	1.13	13.5
16	ICC 3946 ^A	19.68	57.2	103.2	3946	1341	34.1	1486	1.15	11.4
17	ICC 15612	19.68	43.9	87.3	3616	1714	48.9	1463	1.02	15.5
18	ICC 440	19.67	54.9	100.2	4052	1821	45.1	1404	1.22	11.7
19	ICC 1098 ^A	19.64	49.8	94.5	3971	1975	50.8	1716	1.12	13.4
20	ICC 9002 ^A	19.63	49.3	93.9	3420	1710	51.8	1385	1.27	12.1
<i>Low $\Delta^{13}C$</i>										
1	ICC 10500	18.22	52.7	97.4	4506	1681	37.1	1092	0.98	21.5
2	ICC 10885	18.21	55.7	104.7	4237	1232	27	650	0.88	28.4
3	ICC 3761	18.21	45.7	90.6	3705	1508	40.9	1504	1.2	10.8
4	ICC 1052 ^A	18.21	48.3	96.9	3642	1255	34.8	1152	1.37	11.4
5	ICC 12379	18.21	54.6	105.5	4435	1352	29.6	857	1.01	27.6
6	ICC 11303	18.2	49.4	100.6	4217	920	21.6	613	1.05	40.5
7	ICC 8752	18.19	52.5	103.6	4118	1141	27.1	1053	1.08	14
8	ICC 6875	18.18	53.3	99.9	4212	1263	29.4	945	0.99	18.6
9	ICC 13599	18.11	51.9	98.2	3844	1407	36	957	1.03	19.7
10	ICC 8855	18.1	41.9	90.9	3920	1813	47.8	1427	1.07	17
11	ICC 11903	18.08	53.8	106.9	4128	1274	29.6	979	0.92	26
12	ICC 2990 ^A	18	52.6	103.9	3759	1329	36.2	1032	1.01	17.6
13	ICC 8515	17.91	60.4	112.2	4641	1144	23	959	1.07	15.4
14	ICC 7819 ^A	17.86	50.6	99.8	4123	1497	36.3	1189	1.01	21.1
15	ICC 12028	17.81	51	99	4168	1489	35.7	1178	1.05	19.6
16	ICC 9712	17.73	51.1	96.2	4454	1542	34	1391	1.13	14.6
17	ICC 7554	17.59	51.5	99.5	4049	1300	32.4	953	1	21.9
18	ICC 6905	17.58	54.6	107.8	4690	970	18.9	736	0.93	25.5
19	ICC 6293	17.53	51.1	100.9	3984	1362	34.2	1140	1.24	11.2
20	ICC 3239	17.51	55.1	108.8	3445	871	24.7	787	1.02	15
21	ICC 10569	17.45	52.1	100.4	3448	1261	35.9	887	1.25	18.7

^AEntries appear as drought tolerant and highly sensitive ones in work by Krishnamurthy *et al.* (2010).

area, and short growth duration. With a similar view, Sinclair (2012) described TE as a difficult trait to resolve (owing to its high dependency upon physiological and environmental variables) for use in breeding programs and proposed a direct use of component traits that contribute to TE. However, it has also been demonstrated in a large set of sorghum germplasm, where water use and WUE were assessed gravimetrically in lysimeters, that WUE bore no significant relationship with total plant water use, indicating that selection for both high WUE and high water use (Vadez *et al.* 2011). The fact that a

most of the high WUE accessions of this study also produced consistently the best drought yields (across 3 years) in severely water-limited environments (Krishnamurthy *et al.* 2010) overrules these concerns of poor water use or less biomass production as far as the receding soil moisture environments of Patancheru is concerned. Moreover, selection for both $\Delta^{13}C$ and high photosynthesis had been shown to be achievable with a clear 10% yield advantage in wheat by following a dual selection approach (Rebetzke *et al.* 2002). This had been practiced by first selecting lines on stomatal conductance, thereby eliminating

Table 3. Correlation coefficients of carbon isotope discrimination ($\Delta^{13}\text{C}$) with days to 50% flowering, days to maturity, shoot biomass (kg ha^{-1}), seed yield (kg ha^{-1}), HI (%), pods (number m^{-2}), seeds pod^{-1} and 100 seed weight (g) across 2008–09 and 2009–10 seasons under both drought stressed (DS) and optimally irrigated (IR) environments

These correlations were performed using the means of all the 280 accessions. Significance levels are indicated: *, $P < 0.05$; **, $P < 0.01$

Year/irrigation	Days to 50% flowering	Days to maturity	Shoot biomass (kg ha^{-1})	Seed yield (kg ha^{-1})	Harvest index (%)	Pod number m^{-2}	100 seed weight (g)
2008–09, DS	-0.014	-0.148*	-0.167**	0.253**	0.423**	0.416**	-0.318**
2008–09, IR	-0.076	-0.133*	-0.176**	0.232**	0.413**	0.283**	-0.135**
2009–10, DS	-0.448**	-0.498**	-0.505**	0.513**	0.694**	0.465**	-0.291**
2009–10, IR	0.116*	0.014	-0.162**	0.01	0.104**	0.088	-0.046
Both years, SE	-0.223**	-0.339**	-0.431**	0.377**	0.569**	0.485**	-0.350**
Both years, IR	-0.038	-0.121	-0.254**	0.193**	0.359**	0.226**	-0.075
Both years, both irrigations	-0.195**	-0.300**	-0.441**	0.364**	0.558**	0.459**	-0.268**

Table 4. Path coefficients showing the direct and indirect associations (through days to 50% flowering, days to maturity, shoot biomass (kg ha^{-1}), HI (%), pods (number m^{-2}) and 100 seed weight (g)) of carbon isotope discrimination ($\Delta^{13}\text{C}$) with seed yield during the 2008–09 and 2009–10 seasons under both drought stressed (DS) and optimally irrigated (IR) environment

These path coefficients are arrived using the means of all the 280 accessions

Year/irrigation	$\Delta \Delta^{13}\text{C}$	Days to 50% flowering	Days to maturity	Shoot biomass (kg ha^{-1})	Harvest index (%)	Pod number m^{-2}	100 seed weight (g)	Seed yield (kg ha^{-1})
2008–09, DS	-0.007	-0.001	0.002	-0.143	0.406	-0.004	0.001	0.253
2008–09, IR	-0.001	0.001	-0.001	-0.134	0.372	-0.012	0.006	0.232
2009–10, DS	-0.03	-0.014	-0.015	-0.335	0.819	0.006	0.005	0.513
2009–10, IR	-0.006	0.002	0	-0.092	0.1	0.004	0.001	0.01
Both years, DS	0.044	-0.005	-0.001	-0.271	0.609	-0.002	0.004	0.377
Both years, IR	0.001	0.001	-0.003	-0.164	0.355	-0.002	0.005	0.193
Both years, both irrigations	0.028	-0.006	-0.017	-0.28	0.624	0.005	0.01	0.364

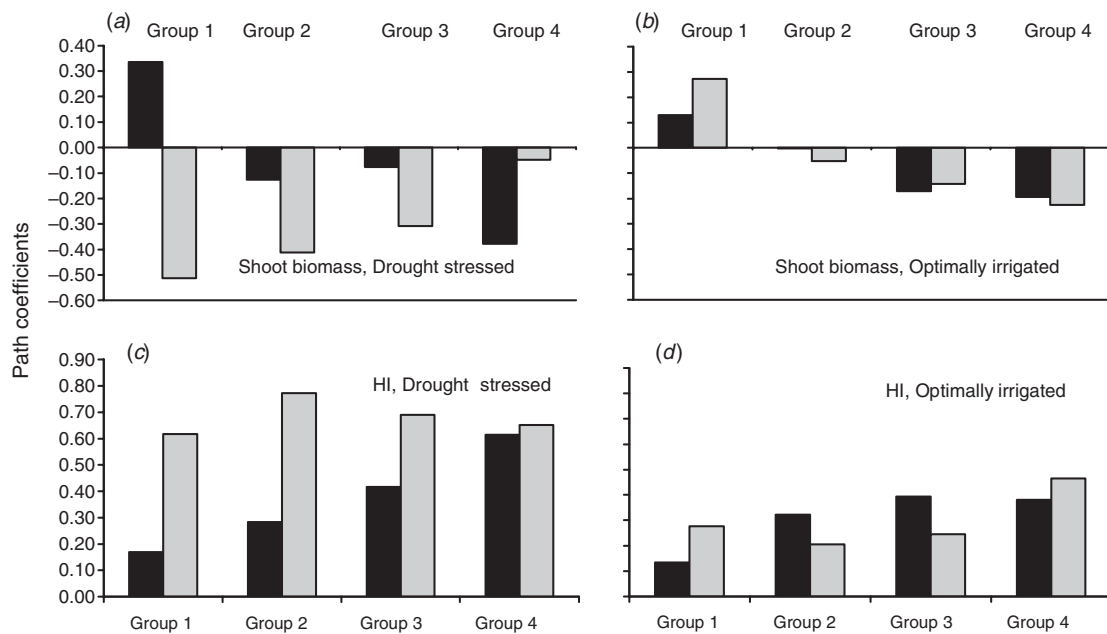


Fig. 4. Association of carbon isotope discrimination with seed yield through (a) shoot biomass in all the four phenological groups under drought stressed (DS) condition, (b) optimally irrigated (IR) condition and through (c) harvest index (HI) in all the phenological groups in the drought stressed (DS) condition (D) and optimally irrigated (IR) condition. Black bars are the associations during 2008–09 and grey bars are during 2009–10.

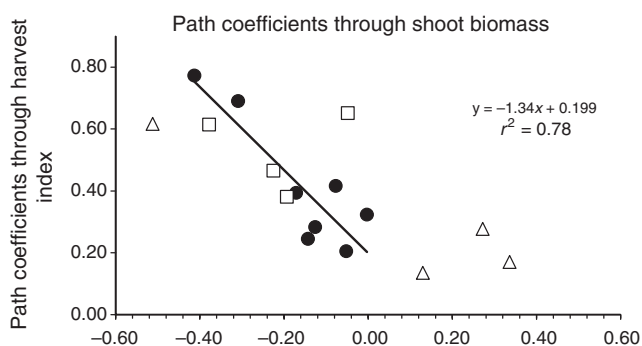


Fig. 5. The relationship between the path coefficients of shoot biomass and the harvest index (HI) in the first group (open triangles), second and third group (closed circles) and the fourth group (open squares). The regression coefficient and the trend line are specific to the second and third group points only.

those with poor photosynthetic performance and selecting for WUE in a second step. Simultaneously, there is a need to look for variations that are non-physiological in nature but influence $\Delta^{13}\text{C}$ and yield such as leaf glaucousness in barley (Febrero *et al.* 1998) and early flowering and maturity in *Arabidopsis* (Mckay *et al.* 2003).

$\Delta^{13}\text{C}$ and its relationship with yield and other yield components

The greatest challenge in using $\Delta^{13}\text{C}$ in breeding for greater agronomic WUE is the high level of inconsistency observed in the relationship between $\Delta^{13}\text{C}$ and yield (Condon *et al.* 2004). Such conflicts of $\Delta^{13}\text{C}$'s association with yield seem to be influenced by soil moisture environments of the study (Rowland and Lamb 2005; Tardieu 2012). $\Delta^{13}\text{C}$ is a product of differences that are largely influenced by the variations in stomatal conductance and the photosynthetic capacity (Condon *et al.* 2002). When soil moisture levels are decreased, plants commonly respond by simultaneously decreasing photosynthesis, transpiration and leaf conductance (Farquhar *et al.* 1989) and causing variation in intercellular CO_2 concentration/atmospheric CO_2 concentration ($C_i:C_a$) ratios. Large improvements in intrinsic water use efficiency (or $\Delta^{13}\text{C}$) are theoretically possible for relatively modest reduction in the value of $C_i:C_a$. Also, considerably long hours of stomatal closure are required for the integration of carbon discrimination effects and to be apparent. If the leaf conductance decreases at a faster rate under stress than the photosynthetic dependence on intercellular CO_2 concentration, then intercellular CO_2 concentration will decrease affecting $C_i:C_a$ ratios. This effect is measurable as a decrease in $\Delta^{13}\text{C}$. Therefore variable $\Delta^{13}\text{C}$ is possible only when the plant leaves were exposed to adequately longer periods of critical water deficits. Development of water deficit is dynamic and growing crops under terminal drought stress need or need not develop drought stress of the required intensity all through the growing period. Even in crops that are grown under receding soil moisture conditions, at least in the early growth stages in winter, the vapour pressure deficits are at the lowest, the appearance of drought stress is too low to affect stomatal conductance. However, the stress can develop gradually and become severe

with the extent of delays in maturity (Krishnamurthy *et al.* 2010). As a consequence the accessions that flower from 35 to 71 days after sowing experience increasing intensities of drought with the increase in flowering time; the drought intensities vary from drought escape in extra-early and early entries to extreme suffering by the late duration entries. Thus the accessions in reality may complete their life cycle under varying drought intensities and durations and exhibit a range of $\Delta^{13}\text{C}$'s association to yield. Therefore, the characteristic of the final response curve of any group of accessions depends upon both the genetic variation in stomatal conductance (Condon *et al.* 2004) under drought and the quantum of exposure to drought stress. As the absence any one of the parameters can lead to the absence of a response curve (Tardieu 2012), the chances of noticing a response curve is 1 : 4. Therefore, even an occasional appearance of a response is enough to claim the useful association of $\Delta^{13}\text{C}$.

The broad sense heritability values recorded in this study were high, as these field trials were conducted by keeping under check environmental heterogeneity by summer soil sterilisation, choice of homogenous fields and control against biotic stresses. The heritability of times to flowering and maturity, 100 seed weight and the HI were high whereas it was moderate for the total shoot biomass, yield and pod number m^{-2} . The heritability of $\Delta^{13}\text{C}$ was as good as the yield with a value of 0.72 under DS and comparable to the value of 0.8 in a recent successful study that identified major QTL for $\Delta^{13}\text{C}$ in barley (Chen *et al.* 2012) meriting consideration in selections. This study had shown that $\Delta^{13}\text{C}$ associates to seed yield indirectly, both through the total shoot biomass or HI, and considerably under the adapted accessions. TE is negatively related to transpiration and positively to HI (Blum 2005). The path coefficients had indicated that the extent of negative association through shoot biomass brings in a more close positive association through HI (Fig. 5). Finally, the closer association of HI is seen on the seed yield as also shown in other crops (Condon *et al.* 2004). This relationship suggests the existence of a close linkage between $\Delta^{13}\text{C}$ -induced shoot biomass and HI. Therefore, it is quite likely that breeding for $\Delta^{13}\text{C}$ involves linkage drags, and thus, molecular markers that are common to both $\Delta^{13}\text{C}$ and HI or shoot biomass and HI could appear. For example, correlation coefficients calculated using major chunk of the accessions ($n=243$ to 254) showed $\Delta^{13}\text{C}$ to be positively correlated with HI in all moisture environments ($r=2008-09$ DS -0.40 , IR -0.41 , 2009-10 DS -0.64 and IR 0.16).

Conclusions

In this work we measured a large range of variation for $\Delta^{13}\text{C}$ in the reference collection of chickpea germplasm that was wider under drought stress than under optimal irrigation. Drought stress decreased $\Delta^{13}\text{C}$ values compared with optimal irrigation. $\Delta^{13}\text{C}$ was correlated with seed yield more strongly under drought stress and the poor relationship was due to less exposure to drought stress. $\Delta^{13}\text{C}$ contributed to seed yield, indirectly and negatively through total shoot biomass and positively through HI. Selection for greater $\Delta^{13}\text{C}$ or TE balanced the shoot biomass production and HI to produce the best seed yields under drought stress. The concern that selection for higher TE would

automatically mean selections for lesser shoot biomass is valid, and to overcome these, two layers of selection are needed: one for higher $\Delta^{13}\text{C}$ and the other for shoot biomass; when selecting for this trait.

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