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Growth and sucrose synthase activity of developing chickpea (*Cicer arietinum* L.) seeds under field conditions

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Abstract

Seed growth characteristics and sucrose synthase activity in chickpea (*Cicer arietinum* L.) were examined in a field experiment at Merredin, Western Australia. 'Sona', a small-seeded desi cultivar, and 'Kaniva', a large-seeded kabuli cultivar, were grown after flowering with irrigation and under a rainout shelter, used to induce terminal drought. Seed and pod wall dry weight followed a similar pattern in the two cultivars with terminal drought significantly reducing the dry weight of the pod wall and seed in both cultivars. The pod wall reached its maximum dry weight 21 days after podding (DAP) in 'Kaniva' and 28 and 35 DAP in 'Sona' with terminal drought and irrigation, respectively. The dry weight of the pod wall decreased during seed filling, particularly in the plants subjected to terminal drought. The increase in seed dry weight followed a sigmoid curve with a lag phase of 14 DAP and 21 DAP in 'Kaniva' and 'Sona', respectively, followed by a rapid almost-linear phase until 35 DAP when the dry weight leveled off and even decreased slightly near maturity. Sucrose synthase activity peaked at 32 DAP in 'Kaniva' and 35 DAP in 'Sona' and then decreased to near zero at maturity. A significant and positive association was observed between seed dry weight at maturity and peak sucrose synthase activity in both cultivars and both treatments. We suggest that sucrose synthase is a good physiological indicator for use in breeding for improved seed size in chickpea.

***Key words*: s**eed growth; water stress; pod wall; seed coat; cotyledon; remobilization; terminal drought

Introduction

Chickpea is subjected to terminal drought both in the winter-rainfall Mediterranean-climatic regions where it grows on current rainfall and in the summer-rainfall sub-tropical regions where it grows on stored soil moisture. As a consequence of the terminal drought, seed yield and quality of chickpea can be drastically reduced. Production of dry matter, early vigour, phenological plasticity and osmotic adjustment have been identified as some of the key characteristics for improved crop yields in water-limited environments (Turner, 1997). In chickpea, genotypic differences for leaf photosynthesis, dry matter accumulation and redistribution, osmotic adjustment, rate and duration of seed filling have been observed (Davies *et al.*, 1999; Leport *et al.*, 1999), but have not been reliably related to yield under terminal drought (Leport *et al.* 1999; Basu *et al.*, 2007; Turner *et al.*, 2007). Low leaf photosynthetic rates (Singh *et al.*, 1987) during seed filling are thought to be the major cause of reduced seed size with water shortage (Leport *et al.*, 1998). However, these studies did not determine the ability of sink tissues to receive and to metabolize imported assimilates from the source tissues. After cellular division in the endosperm and the beginning of the linear phase of cotyledonary growth, utilization of sucrose for starch synthesis dominates. As plant sinks grow, sucrose is hydrolyzed either by sucrose synthase or by an acid invertase depending on the stage of endosperm development (Huber and Akazawa, 1986; Sung *et al.*, 1988; Xu *et al.*, 1988;

Table 1. Dry weight of the seed coat at its maximum and at maturity and dry weight of the two cotyledons per seed at maturity in two chickpea cultivars, 'Sona' and 'Kaniva', with irrigation and terminal drought. 'Kaniva' attained maximum seed coat weight 21 days after podding (DAP) and 'Sona' 28 DAP.

Treatments/Cultivar	Seed coat (mg seed^{-1})		Cotyledons at maturity (mg seed^{-1})
	Maximum	Maturity	
Irrigation			
'Sona'	73.2 c	76.3 d	190.8 b
'Kaniva'	70.8 c	35.5 b	370.0 c
Terminal drought			
'Sona'	49.8 a	51.6 c	177.3 a
'Kaniva'	60.3 b	31.3 a	347.6 d

Means followed by the same letter within columns are not significantly different (P<0.01)

Weber *et al.*, 1996; Weber *et al.*, 2005). Sung *et al.* (1994) observed that invertase activity and sucrose import coincided closely only during pod elongation and early dry weight accumulation in seeds of *Phaseolus vulgaris* L. However, acid invertase activity did not change appreciably during seed development (Riffkin *et al.*, 1995; Turner *et al.*, 2008). Moreover, sucrose synthase activity is reported to be strongly correlated with seed and fruit growth in several crop species (Sung *et al.*, 1989, 1994; Xu *et al.*, 1989; Sun *et al.*, 1992; Weber *et al.*, 1998; Turner *et al.*, 2008), and it is the major enzyme hydrolyzing sucrose during the linear phase of seed growth (Jennings and Morton, 1962; Chevalier and Lingle, 1983; Turner *et al.*, 2008). Winter and Huber (2000) have reviewed the work on regulation of sucrose metabolism in higher plants, but only a few reports are available on dicotyledons. As seed size and uniformity determine the market price of chickpea, particularly kabuli chickpea, identification of the factors maintaining seed size, particularly in water-limited conditions, will be important in breeding programs aimed at improving the size of chickpea. This study was carried out to investigate the seed growth characteristics of two important chickpea genotypes, one a small-seeded desi and one a large-seeded kabuli, under adequately- watered conditions and terminal drought, and to clarify whether sucrose synthase is involved in sucrose metabolism and the rate and duration of seed growth in chickpea.

Materials and methods

Two cultivars of chickpea (*Cicer arietinum* L.), the small-seeded (0.19 g seed^{-1}) desi cultivar 'Sona' and the large-seeded (0.36 g seed^{-1}) kabuli cultivar 'Kaniva' were grown in a fine textured soil (Calcic Haploxeralf) in the field at the Merredin Research Station of the Western Australian Department of Agriculture and Food, Merredin, Western Australia (31°30'S, 118°12'E). Details of the soil at the site are given in Thomson *et al.* (1997). The plots were sown on 1 June in plots 6 m by 1.08 m (6 rows, 180 mm apart) to give a final density, measured 68 days after sowing (DAS), of 45 plants m^{-2}. The two cultivars were replicated three times with each cultivar randomly allocated to two watering regimes: (i) irrigated by drip irrigation twice weekly from flowering (90 DAS) to 145 DAS to replace water lost by evapotranspiration, as measured by pan evaporation, and (ii) maintained from flowering to maturity (157 DAS) under a rainout shelter that covered the plots whenever rain threatened in order to induce terminal drought, At the site, an automatic weather station recorded daily maximum and minimum air temperature, relative humidity, while rainfall and pan evaporation were measured manually.

Pod and seed growth and sucrose synthase activity in the cotyledons was followed from podding (110 DAS) to maturity (157 DAS) in the two chickpea cultivars exposed to the two watering treatments.

Pod and seed growth

First flowering was recorded twice weekly in each plot and, when sufficient pods were just visible (about 2 mm in length), a minimum of 60 pods per plot were tagged; the date of tagging was referred to as 0 day and subsequent days as days after podding (DAP). Three tagged pods per plot were harvested ten times from tagging to maturity. After harvest, the pods were separated into pod wall and seed for dry weight determination after oven drying to constant weight at 65°C. The seed coat and cotyledons were also separated at 21 DAP in 'Kaniva' and 28 DAP in 'Sona' and the dry weights determined after oven drying to constant weight at 65 °C.

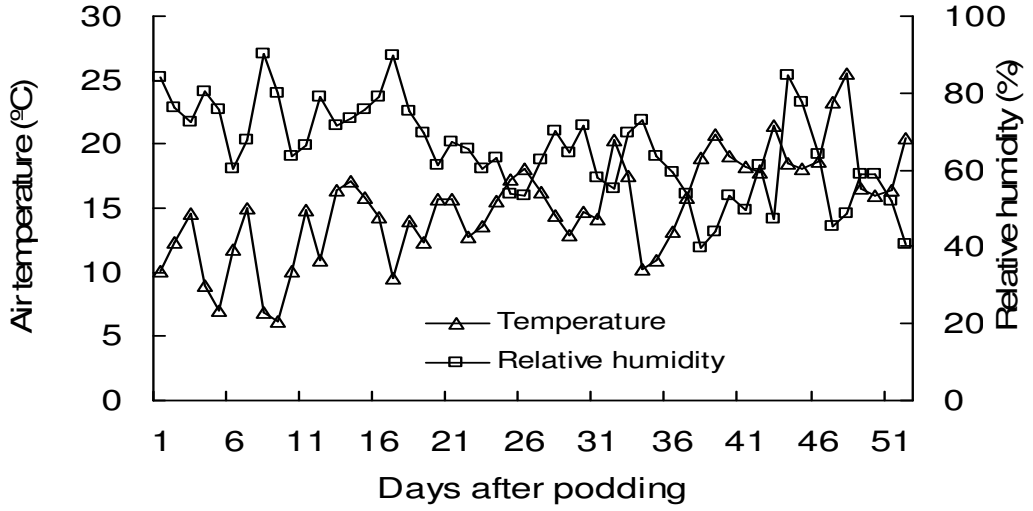

Fig 1. Daily mean air temperature (Δ) and relative humidity (□) during the study period at the experimental site in Merredin, Western Australia.

Enzyme assay

Sampling for sucrose synthase activity was done on the same occasions as those for the measurement of dry weight. Three pods per plot were harvested, seed (s) taken out and the cotyledons separated. One cotyledon per pod was sampled into a micro test tube and immediately frozen in liquid nitrogen. In the laboratory, the frozen cotyledon samples were thawed and ground in the micro test tube with a micro pestle. One ml of extraction buffer (1g polyvinylpyrolidone (PVP) dissolved in 100 ml of stock buffer containing 50 mM N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid (HEPES), 8 mM $MgCl_2$, 2 mM ethylenediaminetetra-acetic acid (EDTA), 50 mM Mercapto and 12.5% glycerol dissolved in 500 ml of distilled water and pH adjusted to 8 with trizma base) was added, vortexed and the sample kept on ice. When all the samples were processed, they were centrifuged in a micro centrifuge at 13,000 rpm (28,340g) for 120 s. As much as possible of the supernatant (about 0.9 ml) was pipetted on to a cation exchange column (Column PD-10, Sephadex G25M Columns, Pharmacia Biotech) after which the column was washed three times with distilled water and then three times with stock buffer (pH 8). When the supernatant had passed through the column, 1.6 ml of extraction buffer was added on to the column and allowed to pass through.

Then 3.5 ml of extraction buffer was pipetted on to the column and the filtrate was collected in glass vials and the samples stored on ice. One hundred µl of filtrate was added to a micro test tube containing 25 µl 40 mM uridine 5'-diphosphate glucose (UDPG) and 50 µl 40 mM fructose, vortexed and the sample incubated at 30°C for 900 s. After incubation, the mixture was neutralized by adding 175 µl 1N NaOH. At the same time, 100 µl of sample extract was added to a second micro test tube containing 25 µl 40 mM UDPG, 50 µl 40 mM fructose and 175 µl 1N NaOH. The reaction in both test tubes was then stopped by boiling the sample for 600 s. Samples were cooled down and 250 µl resorcinol (0.1% w/v) in 95% ethanol and 750 µl concentrated HCl were added. Samples were floated in a water bath at 80°C for 480 s along with a set of standards. The standards were prepared for 0, 100, 200, 300, 400 and 500 nmol sucrose. Before putting the standards in the water bath at 80°C, 250 µl resorcinol and 750 µl concentrated HCl were added. Absorbance was measured at 520 nm optical density in the samples that were incubated at 30°C for 900 s and those not incubated to determine the sucrose synthase activity by difference (Kumar and Turner, 2002). The catalytic activity of sucrose synthase is given in katals where 1 katal raises the rate of reaction by 1 mol s^{-1}.

Statistical analysis

The dry weights and sucrose synthase activity from the three pods per plot were averaged before statistical analysis in factorial randomized blocks with two factors (irrigation and cultivar), two levels for each factor and three replicates. Averaged values were subjected to analysis of variance (ANOVA) for each characteristic by following Online Statistical Analysis (OPSTAT, www.hau.ernet.in).

Results

During the period after tagging, the average daily temperature ranged between 6.2 and 25.5°C and average daily relative humidity between 40 and 90% (Fig. 1). The minimum temperature fell below 0°C on three occasions during the period of sampling, while the maximum temperature reached above 35°C on one occasion near maturity. During the period of sampling the rainout shelter kept 21 mm of rainfall from the stressed plots.

First flowering was 8 days earlier in 'Sona' (87 DAS) than in 'Kaniva' (95 DAS), while pod set started about 10 days earlier in 'Sona' than 'Kaniva'. However, in the study all the pods (<2 mm long) were tagged on the same day (110 DAS) in the two cultivars.

The changes with time in the dry weight of the pod wall, seed and total pod are presented in Figure 2. The general pattern of seed and pod wall growth was similar in the two cultivars. The pod wall grew first and had almost reached its maximum dry weight before seed growth began. Under the rainout shelter and irrigated conditions, respectively, the pod wall dry weight increased to a maximum at 28 and 35 DAP in 'Sona', while it reached peak values at 21 DAP in both environments in Kaniva (Fig. 2A). On average, the peak dry weight values of the pod wall were significantly (P<0.05) greater in the irrigated (139 mg pod^{-1}) than in the stressed (93 mg pod^{-1}) plants and in the large-seeded 'Kaniva' (119 mg pod^{-1}) than in the small-seeded 'Sona' (113 mg pod^{-1}). In both cultivars there was a greater decrease in the pod wall weight in the stressed plants than in the irrigated plants as seed filling proceeded. The decrease was 23.7 mg pod^{-1} in 'Sona' and 37.8 mg pod^{-1} in 'Kaniva', 26 and 40% of the maximum pod wall weight for the two cultivars, respectively. The reduction in pod wall dry weight was less than 10 % in both cultivars in the irrigated treatment.

The pattern of dry weight accumulation followed a similar sigmoid curve in both the seed weight (Fig. 2B) and total weight (Fig 2C). In the seed, there

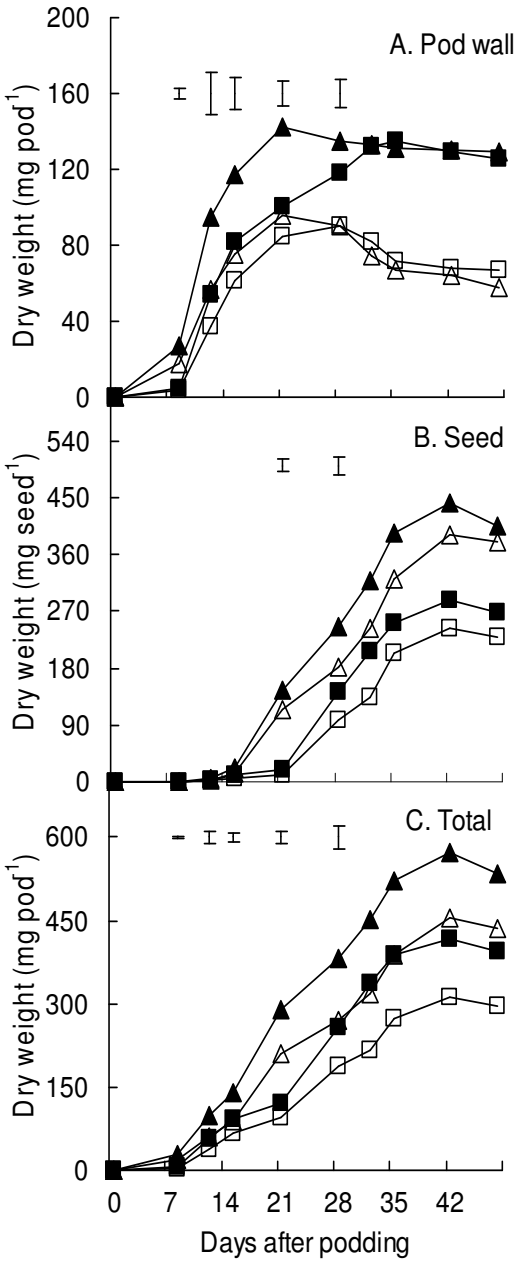

Fig 2. Changes with days after podding in pod wall, seed and total seed dry weight of pods in two cultivars of chickpea, 'Sona' (□, ■) and 'Kaniva' (Δ, ▲) with irrigation (■, ▲) and terminal drought (□, Δ). The vertical bars indicate least significant difference (P<0.01) for the interaction between the cultivars and water treatments when the interactions were significant.

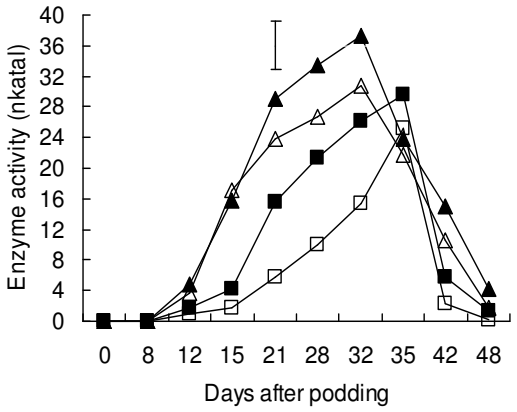

Fig 3. Changes with time after podding in sucrose synthase activity in the cotyledons of two cultivars of chickpea, 'Sona' (□, ■) and 'Kaniva' (Δ, ▲) with irrigation (■,▲) and terminal drought (□, Δ). The vertical bar indicates least significant difference ($P<0.01$) for the interaction between the cultivars and water treatments when the interaction was significant

was an early period (up to 21 days) when there was little increase in dry weight, then a period of rapid seed filling with an effectively linear increase in dry weight (up to 35 DAP), after which the weight gain leveled off and even decreased in the last 6 days (Fig. 2B). The lag phase before the dry weight of the seed began to increase was longer in 'Sona' than in 'Kaniva' in both the stressed and irrigated treatments. The rate of linear growth was slower in the stressed chickpeas than in the irrigated chickpeas, leading to a lower final seed weight in the stressed than irrigated plants in both cultivars (Fig. 2B). In 'Sona' the final seed dry weight in the irrigated plants was 267 mg pod^{-1} compared to 228 mg pod^{-1} in the stressed plants, a 14.6% reduction. The respective values for Kaniva were 405 and 379 mg pod^{-1}, a significant ($P<0.05$) 6.4% reduction. As expected, the seed weight was significantly greater ($P<0.01$) in 'Kaniva' than in 'Sona' in both watering treatments.

The weight of the seed coat was significantly ($P<0.05$) greater in the irrigated than in the stressed treatment in both cultivars at both the maximum weight of the seed coat and at maturity (Table 1). It was also significantly greater in 'Sona' than 'Kaniva' at seed maturity, due to a marked decrease in seed coat weight after the maximum had been reached in 'Kaniva'', but not in 'Sona' (Table 1).

Sucrose synthase activity was very low for the first 12 DAP, reached peak values at 32 DAP in 'Kaniva' and 35 DAP in 'Sona' and then decreased to maturity (Fig. 3). The decrease was more rapid in 'Sona' than 'Kaniva'. Sucrose synthase activity was lower under stress conditions, particularly in 'Sona', but peak enzyme activity was observed at the same time in the two environments (Fig. 3). Peak enzyme activity was significantly higher ($P<0.05$) in the large-seeded 'Kaniva' than in the small-seeded 'Sona'.

There was a positive linear association ($R^2 = 0.70$) between seed dry weight at maturity and the peak values of sucrose synthase activity in both the irrigated and terminally-stressed 'Kaniva' and 'Sona' (Fig. 4).

Discussion

Seed growth followed a sigmoid curve in both the small-seeded desi and large-seeded kabuli chickpea. There was a long lag phase before seed growth commenced, followed by a rapid, almost linear phase of dry weight increase before the rate of growth leveled off and even decreased near maturity. The maximum rate of seed growth was faster and the lag phase was shorter in 'Kaniva' than 'Sona', leading to heavier (larger) seeds in 'Kaniva' than in 'Sona' in both the irrigated and stressed plants. The terminal drought significantly reduced the rate of seed growth in both types of chickpea, as observed by Davies *et al.* (1999), but not the duration of growth. In both cultivars the increase in dry weight of the pod preceded the growth of the seed, reaching a maximum dry weight shortly after the seeds began to grow and then decreasing towards maturity. The decrease was smaller in the irrigated chickpeas (<10%) than in the chickpeas subjected to terminal drought, and smaller in 'Sona' (26%) than in 'Kaniva' (40%), suggesting a greater remobilization of dry matter from the plants subjected to terminal drought than in the irrigated plants.

The seed coat also lost weight after attaining its maximum value in 'Kaniva', but not in 'Sona', suggesting that remobilization of carbon from the pod wall and seed coat may be greater in the kabuli cultivar than in the desi cultivar. Remobilization studies have shown a greater percentage, but not absolute, remobilization of pre-podding carbon and nitrogen to the seed in water-stressed chickpea than in adequately-watered chickpea and in a desi chickpea than a kabuli chickpea (Davies *et al.*, 2000). The greater loss of dry weight from the pod wall in the kabuli cultivar than in the desi cultivar in the present study is consistent with the results of

Davies *et al.* (1999), suggesting that with water shortage during seed filling, the growth of the large-seeded kabuli seed may act as a greater sink for carbon from the pod wall and the seed coat than the small-seeded desi seed. However, Leport *et al.* (2006) cautioned against using dry weight changes as an absolute measure of remobilization in chickpea. The seed growth was strongly associated with the activity of the sugar enzyme sucrose synthase in the cotyledons with final seed weight at maturity being closely associated ($R^2 = 0.70$) with the maximum sucrose synthase activity in both the irrigated and water-limited treatments. This confirms observations in the glasshouse in which the final seed size was positively associated with sucrose synthase activity when the seed growth rate was maximal (Turner *et al.*, 2008). Maximal growth and sucrose synthase activity occurred at 32 to 35 DAP in the present field study, but only 10-14 DAP in the glasshouse (Turner *et al.*, 2008). As the mean temperature in the glasshouse studies was warmer than in the field, the maximal growth seed growth and sucrose synthase activity was calculated to occur at 400 to 600 growing degree days in both environments. In this field study sucrose synthase activity was clearly shown to decrease as seed growth slowed, to reach values near zero at physiological maturity (Fig 3).

The ability of a tissue or organ to synthesize and metabolize sucrose is considered the major determinant of sink strength (Ho, 1988). The rate of synthesis of storage materials in the seed and the length of time that the synthesis continues is controlled by seed itself, as opposed to being controlled by the supply of assimilates from the plant (Chowdhury and Wardlaw, 1978; Poneleit and Egli, 1979). The activity of the enzyme sucrose synthase in the seed cotyledons may, therefore, serve as an indicator of sink strength. The present study suggests that the sink strength of the kabuli chickpea was greater than the sink strength of the desi chickpea and the sink strength of the irrigated chickpea was greater than those subjected to terminal drought, leading to greater accumulation of dry weight in the seeds of the kabuli than desi chickpea and greater dry weight accumulation in the seeds of the irrigated than stressed chickpeas. The slower decrease in sucrose synthase activity in 'Kaniva' than in 'Sona' near physiological maturity may also be linked to the greater sink strength and the greater remobilization of dry matter from the pod wall and seed coat in 'Kaniva' than in 'Sona'. The strong association between sucrose synthase activity and final seed weight in the present study and between sucrose synthase activity and seed size

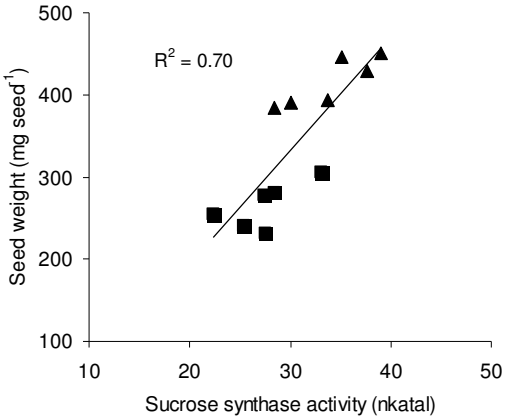

Fig 4. Relationship between seed dry weight at maturity and peak sucrose synthase activity in two chickpea cultivars, 'Sona' (■) and 'Kaniva' (▲), with irrigation and terminal drought.

(Turner *et al.*, 2008) in both irrigated and water-limited environments suggests that sucrose synthase activity is a desirable attribute to be conserved in the gene pool. As seed size is a major quality determinant in the market, the conclusion that seed dry weight accumulation, seed size and sucrose synthase activity are controlled by a major gene or genes (Turner *et al.*, 2008) makes selection of high sucrose synthase activity a desirable breeding objective. As the measurement of sucrose synthase activity is not routine, the identification of a molecular marker to aid in marker-assisted selection of the trait is desirable.

Conclusions

The strong association between sucrose synthase activity during rapid seed filling and final seed dry weight accumulation, and hence seed size, suggests that sink strength is an important determinant of seed size in chickpea. Sink strength, as determined by sucrose synthase activity, varied with genotype and with water availability during seed filling. Water shortage reduced the activity of the enzyme and seed size in both the large-seeded kabuli and the small-seeded desi cultivars, but the higher enzyme activity in the large-seeded kabuli, particularly during late seed filling, appeared to induce greater remobilization of assimilates from the pod wall and seed coat. The higher sucrose synthase activity in the cotyledons is considered

important in breeding for improved seed size in chickpea irrespective of the growing environment.

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