Chilling effects during seed filling on accumulation of seed reserves and yield of chickpea

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Abstract: Chilling (<15 °C) during the reproductive phase of chickpea leads to abortion of flowers and pods, infertile pods, smaller seeds and reduced seed yields. In the present study, effects of chilling during seed development were evaluated on accumulation of seed reserves and yield parameters in an extra early maturing chickpea genotype ICCV 96029. Relative to control plants (17/28 °C mean minimum/maximum temperature), those subjected to cold stress (5/13 °C mean minimum/maximum temperature) showed a marked increase in electrolyte leakage, while cellular respiration (assessed as 2,3,5-triphenyl tetrazolium chloride reduction activity), chlorophyll content, relative leaf water content and rate and duration of seed filling decreased significantly. In cold-stressed plants, seed number per 100 pods, seed weight per plant, average seed weight and average seed size decreased by 35, 43, 41 and 24% respectively. Seed reserves of starch, protein and fat decreased by 34, 33 and 43% respectively, while total soluble sugars increased twofold. The accumulation of storage proteins such as globulins and albumins was inhibited to a greater extent than that of prolamins and glutelins. Most of the amino acids decreased as a result of stress, while some such as proline and glutamic acid increased significantly. Among the minerals examined, phosphorus content decreased more than calcium and iron contents.

Keywords: chickpea; chilling; seed composition; starch; proteins; yield

INTRODUCTION

Chickpea (Cicer arietinum L) ranks first in both area and production amongst pulses in India, which accounts for about 75% of the world’s production. Being a major vegetable source of protein, it occupies a prime position in the staple diet of India. It is cultivated during the winter season in northern parts of India, where it experiences chilling temperatures (<15 °C) during the reproductive phase. The reproductive phase in chickpea includes bud, anthesis, pod set, pod-filling and maturity stages.1 Each stage may show differential sensitivity to chilling temperatures that consequently determine the production potential of the crop.2 At the bud and flowering stages, chilling results in abortion of a large number of these reproductive structures, which has been attributed to male and/or female gamete failures.2 Poor pod set and pod abortion ensue under these conditions, resulting in infertile and fewer pods.3 The relative sensitivity of the pod-filling phase in chickpea to low temperatures is indicated to be higher, but its resultant effects on seed development and composition are not yet known. Early maturing genotypes of chickpea are said to be particularly sensitive to chilling.4

Abiotic stresses occurring during seed development have a profound effect on quantitative and qualitative aspects of seeds.5 There are several reports indicating their inhibitory effects on seed-filling processes, eg drought in the case of wheat,6 soybean7 and chickpea8 and high temperature in the case of maize9 and wheat.10 Comparatively fewer observations exist on effects of cold stress on these aspects, especially in pulses,11 while no information is available in the case of chickpea. The adverse effects of these abiotic stresses on seed development have been associated with inhibition of photosynthesis,12 senescence of source leaves,13 hormonal imbalance,14 restriction in uptake of assimilates into seeds,9 metabolic dysfunction in source or sink organs9,15 and vascular disturbances.16

The present study was conducted with a view (i) to assess the impact of chilling temperatures in the field (5/13 °C mean minimum/maximum temperature) during the seed-filling phase on yield and seed composition as well as (ii) to investigate the possible causes of chilling injury.

MATERIALS AND METHODS

Raising of plants

An extra early maturing chickpea genotype ICCV 96 029 was raised in earthenware pots (30 cm height, 25 cm diameter, 14.721 volume) containing a mixture
of air-dried soil, sand and farmyard manure in a ratio of 2:1:1 (v/v). The soil was loam with a pH of 7.1 and available N, P and K at 54, 43 and 158 kg ha\(^{-1}\) respectively. The seeds were inoculated with *Rhizobium ciceri*\(^{17}\) at the recommended rate of 1.95 g kg\(^{-1}\) seeds. Four seeds were planted in each pot in November, and after emergence the plants were thinned to two per pot. The plants were grown in warm conditions in a glasshouse (17/28 ± 2 °C mean minimum/maximum temperature, 1350 μmol m\(^{-2}\) s\(^{-1}\) light intensity, 60–65% relative humidity) until initiation of seed filling (pod size ~1 cm). Thereafter 100 plants were moved to chilling conditions in the field (5/13 ± 2 °C mean minimum/maximum temperature) for subsequent seed development.

**Evaluation of chilling injury**

Chilling injury was examined in the topmost leaves in terms of electrolyte leakage (EL)\(^{18}\) and cellular respiration (as 2,3,5-triphenyl tetrazolium chloride (TTC) reduction activity). For EL determination, leaves were washed with deionised water to remove surface-adhered electrolytes. The washed leaves were placed in closed vials containing 10 ml of deionised water and incubated at 25 °C on a rotary shaker for 24 h; subsequently the electrical conductivity of the solution (\(L_1\)) was determined. Samples were then autoclaved at 120 °C for 20 min and the final electrical conductivity (\(L_2\)) was measured after equilibration at 25 °C. EL was defined as follows:

\[
\text{EL} \,(\%) = \frac{(L_1/L_2)}{100}
\]

TTC reduction activity was determined according to Steponkus and Lamphere.\(^{19}\) For each treatment (control and cold stress), six replicates were used. The leaves collected from control and chilled plants were washed three times with sterile distilled water and blotted with filter paper. Three replicates of each treatment were incubated for 2 min in 1 ml of water and blotted with filter paper. Three replicates of each treatment were incubated for 2 min in 1 ml of water and incubated at 25 °C on a rotary shaker for 24 h; subsequently the final electrical conductivity (\(L_2\)) was measured after equilibration at 25 °C. EL was defined as follows:

\[
\text{EL} \,(\%) = \frac{\text{TTC reduction activity of treated samples}}{\text{TTC reduction activity of control samples}} \times 100
\]

**Relative leaf water content**

Relative leaf water content was measured according to Weatherley.\(^{20}\) The leaf tissues were excised from the seedling and their fresh weight (FW) was recorded. The leaf discs were soaked in distilled water for 2 h in a petri dish. The discs were taken out and surface dried with blotting sheets and their weight (turgid weight) was recorded. Thereafter the discs were oven dried for 24 h at 110 °C and weighed again (dry weight). Relative leaf water content (RLWC) was calculated as follows:

\[
\text{RLWC} \,(\%) = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100
\]

**Chlorophyll content**

Chlorophyll was extracted with 80% acetone from samples of fresh leaves gathered from control and stressed plants. The extract was measured spectrophotometrically at 645 and 663 nm.\(^{21}\)

**Enzyme assays**

Activity levels of enzymes (sucrose synthase, soluble starch synthase and invertase) were assayed from fresh seeds harvested at physiological maturity from the upper part of the plants. For enzyme assays, samples (500 mg, three replications) were homogenised in ice-cold 200 mM HEPES/KOH buffer (pH 7.8) containing 3 mM EDTA Na\(_2\)-2H\(_2\)O, 3 mM magnesium acetate, 10 mM dithiothreitol (DTT) and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged (10 000 \(\times\) g) for 20 min at 4 °C and the supernatant was used directly as enzyme and protein source. The activities of invertase (EC 3.2.1.26), soluble starch synthase (EC 2.4.1.21) and sucrose synthase (EC 2.4.1.13) were assayed according to Xu et al.\(^{22}\) and Sung et al.\(^{23}\) Assays were performed at 25 °C in a final volume of 1 ml.

**Analysis of seed reserves**

Mature seeds of control and stressed plants were examined for analysis of various seed reserves. Soluble sugars and starch were extracted with 95% (v/v) ethanol and 30% (v/v) perchloric acid respectively. Both components were quantified by the phenol/sulfuric acid method of Dubois et al.\(^{24}\) using glucose (Sigma D9434; Sigma, WI, USA) as standard. Ash, crude protein (micro-Kjeldahl, N × 6.25), crude fat, crude fibre and nutrient contents were determined by standard AOAC\(^{25}\) procedures. Sucrose, glucose and fructose were measured by gas chromatography according to the method of Liu and van Staden.\(^{26}\) Protein fractions (albumins, globulins, prolamins and glutelins) were extracted sequentially from seeds according to the method of Tribol et al.\(^{27}\) Briefly, seeds were ground to wholemeal flour. During each extraction step the samples were stirred continuously on a magnetic stirrer for 60 min. Soluble and insoluble fractions were separated by centrifugation at 8000 \(\times\) g for 30 min at the extraction temperature. Albumins and globulins were extracted at 4 °C with 25 ml of 0.05 M sodium phosphate buffer (pH 7.8) and 0.05 M NaCl respectively. Amphiphilic proteins were
extracted at 4°C from the previous pellet with 25 ml of 2% (v/v) Triton X-114, 0.1 M NaCl and 0.05 M sodium phosphate buffer (pH 7.8). Prolamins were extracted at 20°C from the previous pellet with 25 ml of 70% (v/v) ethanol. Glutelins were extracted at 20°C from the previous pellet with 25 ml of 20 g l⁻¹ sodium dodecyl sulphate (SDS), 2% (v/v) 2-mercaptoethanol (2-SH) and 0.05 M tetraborate buffer (pH 8.5). After centrifugation the glutelins were recovered in the supernatant. The protein content of each fraction was determined according to Lowry et al. Amino acid analysis was conducted with an amino acid analysis system following the method of Bourgoin. This involved pre-column derivatisation of the free amino acids with 6-aminquinoline followed by separation on a C18 high-performance liquid chromatograph with fluorescence detection.

Seed growth rate and seed-filling duration
For the investigation of seed growth rate and seed-filling duration, 35 plants growing under control and chilling conditions were examined. Five pods per plant were tagged at the beginning of pod filling (pod size ~1 cm) and followed until physiological maturity of the seeds. Dry weight of the seeds was recorded 7 days after initiation of pod filling and at physiological maturity. The seeds were oven dried at 45°C for 5 days and their weight was recorded. The time (days) required to complete the seed filling was noted in tagged pods.

Yield parameters
For this purpose, 50 plants from each treatment were examined. Seed weight, seed size and number of shrivelled seeds were recorded in 100 pods of each treatment.

Statistical analysis
Observations were replicated thrice and data were analysed for means and standard errors. ANOVA was conducted and critical differences (5% level) were determined between treatments.

RESULTS
A marked increase (60%) in electrolyte leakage (EL) and decrease (68%) in cellular respiration (TTC reduction activity) occurred in cold-stressed leaves (Table 1). Chlorophyll content declined by 34%, leading eventually to necrosis of leaf margins and defoliation of some leaves. Relative leaf water content (RLWC) declined by 23% in stressed plants, indicating development of water stress.

The seed growth rate of stressed plants declined by 43%, while the seed-filling duration was restricted to 14 days compared with 20 days in controls (Table 2). No further growth in size and increment of dry matter occurred thereafter. The seed number per 100 pods declined to 89 relative to 138 in controls. The seed weight per plant, average seed weight and average seed size decreased by 43, 41 and 24% respectively. Of the total seeds per 100 pods, about 69 were below normal size and weight.

The seeds of stressed plants contained 34 and 29% less starch and protein respectively in comparison with controls, while their soluble sugar content doubled (Fig 1). Fat, crude fibre and ash contents declined by 43, 42 and 46% respectively in stressed seeds (Fig 1). Among soluble sugars, sucrose content increased markedly (88%), while glucose and fructose showed little increase (Table 3). The activity levels of starch synthase, sucrose synthase and invertase decreased by 65, 59 and 55% respectively in stressed seeds.

Table 1. Effects of chilling stress during seed filling on electrolyte leakage (EL, %), cellular respiration (2,3,5-triphenyl tetrazolium chloride (TTC) reduction activity, %), relative leaf water content (RLWC, %) and total chlorophyll content (mg g⁻¹ FW) in topmost leaves of control (17/28°C, mean night/day temperature) and stressed (5/13°C, mean night/day temperature) plants. Figures are mean of four replicates and values represent mean ± standard error

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL</td>
<td>13.6 ± 2.3</td>
<td>73.9 ± 3.1ᵃ</td>
</tr>
<tr>
<td>TTC reduction activity</td>
<td>91.2 ± 2.9</td>
<td>23.4 ± 3.4ᵃ</td>
</tr>
<tr>
<td>RLWC</td>
<td>87.4 ± 3.1</td>
<td>64.5 ± 3.7ᵃ</td>
</tr>
<tr>
<td>Total chlorophyll</td>
<td>1.07 ± 0.11</td>
<td>0.71 ± 0.12ᵃ</td>
</tr>
</tbody>
</table>

ᵃ Significant at 5% level.

Table 2. Effects of chilling stress during seed filling on seed yield traits of control (17/28°C, mean night/day temperature) and stressed (5/13°C, mean night/day temperature) plants. Figures are mean of four replicates and values represent mean ± standard error

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed growth rate (mg day⁻¹)</td>
<td>8.1 ± 1.1</td>
<td>4.6 ± 1.5ᵃ</td>
</tr>
<tr>
<td>Seed-filling duration (days)</td>
<td>20.2 ± 1.4</td>
<td>14.1 ± 1.3ᵃ</td>
</tr>
<tr>
<td>Seed number per 100 pods</td>
<td>138 ± 4.6</td>
<td>89 ± 4.1ᵃ</td>
</tr>
<tr>
<td>Seed weight per plant</td>
<td>11.2 ± 1.3</td>
<td>6.4 ± 1.5ᵃ</td>
</tr>
<tr>
<td>Average seed weight (mg)</td>
<td>162 ± 3.4</td>
<td>96 ± 4.1ᵃ</td>
</tr>
<tr>
<td>Average seed size (mm)</td>
<td>6.7 ± 0.8</td>
<td>5.1 ± 0.6ᵃ</td>
</tr>
<tr>
<td>Number of shrivelled seeds (&lt;6.5 mm) per 100 pods</td>
<td>31 ± 2.6</td>
<td>69 ± 1.1ᵃ</td>
</tr>
</tbody>
</table>

ᵃ Significant at 5% level.

Figure 1. Effects of chilling stress during seed filling on composition (% of mature seeds of control (17/28°C, mean night/day temperature) and stressed (5/13°C, mean night/day temperature) plants. Figures are mean of four replicates and values represent mean ± standard error (vertical bars).
Effects of chilling stress during seed filling on amino acid composition (µmol g⁻¹ DM) of whole seeds of control (17/28°C, mean night/day temperature) and stressed (5/13°C, mean night/day temperature) plants. Figures are mean of four replicates and values represent mean ± standard error.

Table 4. Effects of chilling stress during seed filling on soluble sugars composition (µmol g⁻¹ DM) of whole seeds of control (17/28°C, mean night/day temperature) and stressed (5/13°C, mean night/day temperature) plants. Figures are means of four replicates and values represent mean ± standard error.

**DISCUSSION**

The present findings indicated that seed development in chickpea is affected drastically by chilling stress, resulting in a substantial decline in quantitative and qualitative traits of the seeds. Earlier studies on this aspect in chickpea reported a decrease in seed yield in plants cold-stressed during seed development, but the underlying reasons were not investigated. The present findings indicate some of the possible causes that may limit seed development under low-temperature conditions and thus affect seed yield.

Poor seed development in cold-stressed chickpea plants appears to be associated with a decrease in rate and duration of seed filling, which may be attributed to various reasons. Seed filling is primarily dependent upon availability of assimilates received from the leaves, so any environmental factor affecting the production, mobilisation and utilisation of assimilates would restrict seed development. In the present study we observed a severe loss of chlorophyll coupled with electrolyte leakage, indicating damage to chlorophyll and membranes that may occur as a result of chilling-induced photo-oxidation. Consequently, photosynthetic ability may be markedly affected, thus impairing the production of photoassimilates. Chilling may also affect photosynthesis directly by inhibiting the electron transport chain and carbon fixation processes, thus reducing sucrose generation. In legumes the growth rate of seeds has been reported to be reduced if the photosynthetic activity is not sufficient to fulfill the assimilate demand of filling seeds. It has also been observed that, under chilling conditions, growth and carbon export from the leaves often decline more than photosynthesis, resulting in accumulation of carbohydrate, which may inhibit sucrose synthesis in the leaves. This may occur because of mobilisation restrictions exerted at vascular or enzymatic levels. We found compression of xylem and phloem tissues of pod stalks in cold-stressed plants (unpublished), which might put mechanical constraints on the translocation of assimilates to developing seeds. A change in xylem/phloem ratio was noticed in wheat plants subjected to cold stress. Relative leaf water content decreased in our studies, indicating the development...
of chilling-induced water stress,\textsuperscript{37} which may also slow down the movement of assimilates.

Enzymes pertaining to starch and sucrose metabolism examined here showed substantial reduction in their activities in seeds of cold-stressed plants, which might prevent further import of sucrose by the seeds. Sucrose synthesis is restricted at low temperatures because of the high sensitivity of sucrose phosphate synthase to temperature.\textsuperscript{38} We observed relatively higher levels of sucrose in stressed seeds, which may be attributed to its cryoprotective role\textsuperscript{39} or decreased capacity of its utilisation.\textsuperscript{40} Reduced activity levels of sucrose-hydrolysing enzymes, invertase and sucrose synthase, were observed in the present study, suggesting impairment of sucrose utilisation. A starch-synthesising enzyme, soluble starch synthase, also declined, indicating impairment of diversion of hexoses towards starch formation. These findings correspond with earlier studies where enzymes related to accumulation of carbohydrates, such as ADP glucose pyrophosphorylase, glucokinase, sucrose synthase and soluble starch synthase, in maize seeds were reported to be inhibited by temperature stress,\textsuperscript{9} resulting in impaired seed development. The decrease in protein and fat contents observed here is in agreement with findings of Triboi \textit{et al}\textsuperscript{27} and Champolivier and Merrien\textsuperscript{41} respectively. Amino acid composition was altered in stressed seeds, which has been attributed to a change in total quantity of nitrogen.\textsuperscript{42} Certain amino acids such as proline and glutamic acid showed an increase, as noticed previously in seeds of water-stressed chickpea plants.\textsuperscript{8} Proline, like sucrose, has been implicated as a cryoprotective molecule in cold-stressed cells.\textsuperscript{43}

CONCLUSIONS

Our findings suggested that chilling during seed development limits the accumulation of seed reserves of starch, protein, amino acids, sugars and minerals, resulting in a reduction in seed yield. The possible underlying reasons seem to involve chilling-induced loss of chlorophyll, restrictions on mobilisation and/or availability of assimilates and inhibition of activity of enzymes related to starch and sucrose metabolism in developing seeds. Mechanisms of photo-assimilation and seed storage processes under cold stress need further probing in chickpea for any conclusive information on this aspect.

ACKNOWLEDGEMENT

The financial assistance from UGC, New Delhi, India for this research is gratefully acknowledged.

REFERENCES
