High acid invertase activity for a prolonged period in developing seeds/podwall of wild chickpea is detrimental to seed filling

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In the present study factors responsible for low seed biomass in wild Cicer species has been investigated. Cicer judaicum and chickpea cultivar PBG-1 were investigated to compare activities of some enzymes involved in carbon metabolism in podwall and seeds during crop development. Seed filling duration in wild species was about 15 days shorter than that of cultivated varieties due to rapid loss of moisture content and hence resulted in earlier maturity and reduced seed biomass. Longer seed filling duration appeared to be an important factor responsible for greater biomass of chickpea seeds. Because of absence of phosphoenol pyruvate carboxylase from 25-35 days after flowering and low sucrose synthase activities, the podwall of C. judaicum is not in a position to contribute significantly to the sink filling capacity of seeds. High acid invertase, low sucrose synthase activities during seed storage phase cause detrimental effect on seed filling and resulting in highly reduced sink strength and productivity of wild species. Successful transfer of stress tolerance from wild Cicer species to chickpea cultivars need to prevent the transfer of these observed unfavourable biochemical factors so that the productivity of chickpea crop remains unaffected during utilization of wild Cicer species in chickpea improvement.

Keywords: Carbohydrate, Invertase, PEP carboxylase, Seed-filling, Sucrose synthase, Wild Cicer species

Chickpea is the third most important grain legume in the world grown in an area of about 10 million ha with a production of 7.8 million tons. India is a premier chickpea growing country in the world and contributes 75% to the global production. Large population sectors in India and its neighboring countries, many of them vegetarian, depend on chickpea as a main dietary protein source. Chickpea crop is susceptible to a range of biotic and abiotic stresses, which can be devastating to crop yield by about one third every year. Throughout the chickpea production areas the crop is subjected to extremes of temperature and moisture supply and to deficiency or toxicity of minerals in the soil. About 47 diseases and 54 insect pests have been reported for chickpea. A low level of genetic variability within C. arietinum has hampered chickpea breeders in their efforts to minimize the yield losses and to develop widely adapted cultivars with resistance to biotic and abiotic stresses. Breeders are therefore looking into wild relatives of chickpea as an alternative genetic resource for crop improvement. Wild Cicer species have higher level of resistance than the cultivated species for fusarium wilt, botrytis grey mould, leaf miner, bruchid, cyst nematode and cold. Perhaps even more importantly, several accessions of the wild Cicer species are resistant to three or more stresses, whereas no line of C. arietinum has been found to be resistant to more than a single stress. The higher levels of resistance against abiotic and biotic stresses in wild Cicer species might be due to the differential response of the antioxidant system in wild and cultivated genotypes of chickpea.

Wild Cicer species are more tolerant to biotic and abiotic stresses but they have very small seed size. Because of low seed size and productivity of wild species, there is possibility that while transferring genes for stress tolerance from wild into the cultivated varieties, genes determining low productivity are also being transferred. Therefore, there is a need to bifurcate what contributes to their tolerance towards abiotic and biotic stress and what are the biochemical factors which lead to lower sink strength in wild Cicer species. In the present study we have confined ourselves to the factors responsible for lower seed biomass in wild Cicer species. Conversely opposite of it could lead to better sink strength, an important determinant of seed size in chickpea. Metabolism of
carbohydrates is the key event in seed filling. However, there is no information about carbohydrate metabolism in wild *Cicer* species. Therefore we compared the activities of the enzymes of carbohydrate metabolism in podwall and seeds of wild and cultivated varieties of chickpea during crop development to understand why seed mass of wild species is so small.

**Materials and Methods**

Chickpea (*Cicer arietinum* L. cvs. PBG-1) and wild species *Cicer judaicum* (accession no. 182) were raised in the field of Punjab Agricultural University, Ludhiana, following recommended agronomic practices. The crop was sown in November in sandy loam fields in three different plots as replicates. Each plot had 5 rows with 60-70 plants in each row. The plot size was 3.4 × 1.8 m and inter-row spacing was 5 cm. Developing flowers were tagged daily for 5 days at flowering stage and developing pods were collected at 15, 25, 35 and 45 days after flowering (DAF) and brought buried under ice to the laboratory. Pods were separated into podwall and seeds. The fresh samples of podwall and seeds (500 mg) were plunged into 80% ethanol and stored at -20 °C until required for estimation of sugars. Free sugars were extracted twice with 80% ethanol and then twice with 70% ethanol and reducing sugars were estimated. To study biomass accumulation, a known weight of fresh tissue (3 replicates of 5 pods each) was kept at 60 °C in incubator for 2 days and its weight was determined after every 24 h. Drying of samples was continued till constant weight was obtained.

Triplicate fresh samples of podwall and seeds (100 mg) were taken from each plot for enzyme extraction. All extraction procedures were carried out at 4 °C with insoluble polyvinylpyrrolidone (100 mg/g tissue) added to the extraction media. Acid Invertase and alkaline invertase were extracted and assayed. Sucrose synthase, hexokinase and ADPG pyrophosphorylase were extracted by homogenizing the tissues in 20 mM HEPES buffer (pH 8.0) containing 1 mM EDTA, 5 mM MgCl₂ and 5 mM β-mercaptoethanol. For extracting PEP carboxylase, 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM MgCl₂, 5 mM β-mercaptoethanol and 2 mM EDTA. Homogenates of above mentioned enzyme extractions were centrifuged at 10,000 g for 20 min and the supernatant was used for assaying enzyme activities. Sucrose synthase activity was measured by incubating 1 M HEPES buffer (pH 6.5), 4 mM of UDP, 100 mM of sucrose and enzyme extract at 37 °C for 30 min and fructose released was estimated. Activities of ADPG pyrophosphorylase, hexokinase and PEP carboxylase were determined. Protein content was also measured. Statistical analysis has been done using student’s *t*-test.

**Results and Discussion**

Plants of chickpea cultivar PBG-1 were larger than their wild relative. Number of primary and secondary branches was more in PBG-1 as compared to *C. judaicum* (Fig. 1a). Biomass accumulation in the above ground vegetative tissues of PBG-1 was 4-5 times greater than *C. judaicum* (Data was not given). Production of vegetative biomass is positively correlated with seed yield of chickpea because of availability of additional photosynthetic capacity from leaf canopy to meet the increased demands for carbon assimilate for dry matter accumulation in developing seeds. Increased photosynthesis increased pod, seed number and weight while decreased photosynthesis reduced pod, seed number and weight. Hence the higher vegetative growth in PBG-1 results in increased pod and seed biomass, whereas *C. judaicum* showed smaller pod and seed size than PBG-1 due to much lesser growth of vegetative tissues (Fig. 1 a,b,c).

Biomass accumulation in podwall and seeds of *C. judaicum* and PBG-1 showed significant variations in their growth rates during development. Developing podwall of PBG-1 showed high biomass from 15 DAF to 25 DAF and thereafter it was gradually decreased till 45 DAF. The moisture content decreased from 79% to 9% from 15 DAF to 45 DAF, respectively (Table 1). However, in *C. judaicum*, a rapid decrease in moisture content of podwall was observed between 15-25 DAF. At 15 DAF, the moisture content in podwall of wild *Cicer* species was 65%, whereas cultivated variety had 79% moisture content at 15 DAF. Almost complete dehydration was observed in developing podwall of *C. judaicum* at 25 DAF. A similar physiological status of podwall of PBG-1 was observed at 45 DAF stage. Data suggested that the podwall of cultivated species remained active for a longer period in comparison with that of wild species, thus is in a better position to contribute to the seed filling.

Developing seeds of PBG-1 showed a slower increase in biomass of seeds from 15 DAF to 25 DAF.
Fig. 1—comparisons of (a)-vegetative growth of wild and cultivated varieties of chickpea; (b)- fresh pods of wild (15 DAF) and cultivated (25 DAF) varieties of chickpea; (c)- mature seeds of wild and cultivated varieties of chickpea.
and after that a rapid increase in dry matter was observed. Maximum rate of biomass accumulation in seeds was observed between 25-35 DAF in PBG-1 (Table 1). However, in *C. judaicum*, biomass accumulation was complete in developing seeds by 25-30 DAF. Moisture content of PBG-1 developing seeds decreased from 83% to 58% from 15 to 35 DAF and then from 58% to 7% in next ten days (35-45 DAF). But *C. judaicum* seeds showed a rapid decrease in moisture content from 83% to 14% within 15-25 DAF and then from 14% to 9% between 25-35 DAF. Rapid loss of water content resulting in seed desiccation during 15-25 DAF in *C. judaicum* may be the cause of shorter seed-filling period. Egli reported that water uptake is required to increase cell volume. Expansion of seed storage cells depends on a continuous inflow of water and the seed-filling phase will end when water uptake decreases. Cells then stop their metabolic activities due to limited water. Hence the available water inside the cell is an important parameter which determines the duration of the seed filling period\(^\text{[14]}\). Various studies on legume seed development suggested a positive correlation between duration of seed filling period and seed biomass. Seed-filling duration in *C. judaicum* was about 15 days shorter than that of PBG-1 and hence resulted in an earlier maturity and reduced seed biomass in wild *Cicer* species. Data suggested a positive correlation between duration of seed filling period and seed biomass. Therefore, to increase yield of chickpea crop, the seed filling period could be increased.

Activities of the enzymes of carbohydrate metabolism (acid invertase, alkaline invertase, sucrose synthase, hexokinase, PEP carboxylase, ADPG pyrophosphorylase) were compared in podwall of wild and cultivated varieties of chickpea during crop development (Fig. 2). Acid invertase in developing podwall was 3-4 times higher in *C. judaicum* as compared to PBG-1. Acid invertase activity in *C. judaicum* decreased from 15 DAF to 35 DAF. Alkaline invertase activity could not be detected at 15 DAF in the podwall of PBG-1. It developed later on in this variety and declined from 25 DAF onwards with progress of sink filling. However, activity of alkaline invertase was higher in mature podwall of *C. judaicum*. This may be responsible for significantly high reducing sugars in mature podwall of *C. judaicum* in comparison with that of cultivated variety PBG-1 (Table 2).

Sucrose synthase activity decreased from 15 to 25 DAF in PBG-1 podwall. Activity of sucrose synthase could not be detected at 35 and 45 DAF in podwall. In *C. judaicum* podwall, sucrose synthase activity was observed at 15 and 25 DAF. However, sucrose synthase in podwall of PBG-1 was significantly higher than *C. judaicum* podwall at 15 DAF (Fig. 2). This could result in more availability of sugar nucleotides for starch synthesis in podwall of chickpea cultivars that can be utilized for seed filling. It has been reported that during early phase of flowering, starch is accumulated in the podwall which is being utilized to provide carbon to the developing seeds\(^\text{[15]}\). Hence, the increased sink strength of podwall of chickpea cultivars because of high sucrose synthase may lead to better seed filling, whereas, podwall of wild *Cicer* species is not in a position to contribute significantly to the sink filling of seeds. In plants, the cleavage of sucrose molecules by sucrose synthase generates only one, whereas inversion of sucrose by invertase liberates two potential substrate molecules for hexokinase\(^\text{[16]}\). The activity of hexokinase in PBG-1 podwall was significantly higher than enzyme activity

| Table 1—Changes in moisture content and biomass of chickpea genotypes during seed filling  
[Values are mean ± SD of 3 replicates of 5 pods each] |
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<tr>
<td></td>
<td><em>Cicer judaicum</em></td>
<td>PBG-1</td>
<td><em>Cicer judaicum</em></td>
<td>PBG-1</td>
</tr>
<tr>
<td></td>
<td>Podwall</td>
<td>Seeds</td>
<td>Podwall</td>
<td>Seeds</td>
</tr>
<tr>
<td>DAF</td>
<td>FW (mg)</td>
<td>DW (mg)</td>
<td>moisture content (%)</td>
<td>FW (mg)</td>
</tr>
<tr>
<td>15</td>
<td>26.2±3.0</td>
<td>9.1±1.0</td>
<td>65.39</td>
<td>119.8±3.4</td>
</tr>
<tr>
<td>20</td>
<td>18.5±1.4</td>
<td>16.4±0.2</td>
<td>11.11</td>
<td>120.0±2.4</td>
</tr>
<tr>
<td>25</td>
<td>18.1±1.0</td>
<td>17.1±0.4</td>
<td>5.56</td>
<td>123.6±2.0</td>
</tr>
<tr>
<td>30</td>
<td>9.0±0.6</td>
<td>8.5±0.3</td>
<td>5.21</td>
<td>112.6±3.6</td>
</tr>
<tr>
<td>35</td>
<td>8.2±0.3</td>
<td>7.8±0.6</td>
<td>5.03</td>
<td>102.6±1.8</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>37.8±0.8</td>
</tr>
<tr>
<td>45</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>28.7±1.8</td>
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<tr>
<td>maturity at 30 DAF in wild <em>Cicer</em> species</td>
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in wild species. Hexokinase activity was not observed in podwall at 35-45 DAF in PBG-1 and 25-35 DAF in *C. judaicum*. PEP-carboxylase catalyses irreversible carboxylation of phosphoenolpyruvate to yield oxaloacetate and P$_i$. PEP carboxylase activities observed in podwall of PBG-1 at 15 and 25 DAF and *C. judaicum* only at 15 DAF (Fig. 2). Because of higher PEP carboxylase activities for longer period, podwall of PBG-1 fixed CO$_2$ released in the pod cavity by respiring seeds and minimizing respiratory carbon losses, may help in improving considerably the carbon economy of the developing pods and enhancing plant productivity. *C. judaicum* podwall did not show PEP carboxylase activity during whole development and may be responsible for highly reduced productivity of *C. judaicum*. ADPG pyrophosphorylase activity increased in podwall of *C. judaicum* and PBG-1 till maturity. But the increase

Fig. 2—Changes in the activities of (a)-acid invertase; (b)-alkaline invertase; (c)-sucrose synthase; (d)-hexokinase; (e)-PEP carboxylase and (f)-ADPG pyrophosphorylase in podwall of wild and cultivated variety of chickpea. Activity of enzymes is expressed as n moles of product formed/min/mg protein. Data are mean ± SD of 3 replicates. $\nabla$: Maturity at 30 Days after flowering (DAF) in wild *Cicer* species. Enzyme activity was not detected. *Differences significant in comparison with PBG-1 at P<0.01; Student’s t-test.*
activities of ADPG pyrophosphorylase in developing podwall was 3-4 times higher in PBG-1 as compared to C. judaicum (Fig. 2).

Activities of the enzymes of carbohydrate metabolism (acid invertase, alkaline invertase, sucrose synthase, hexokinase, PEP carboxylase, ADPG pyrophosphorylase) were also studied in seeds of wild and cultivated varieties of chickpea during crop development (Fig. 3). The developing seeds of C. judaicum had high acid invertase activity till maturity. However, in PBG-1 seeds the enzyme activity appeared only at 15 DAF i.e. early growth phase of seed filling. In developing legume seeds, invertase and sucrose synthase both are expressed in sink tissues, but the predominant sucrolytic activity varies dependent on the species, tissue type and developmental stage. Acid invertase activity is high during early growth phase of seed development and during rapid phase of sink filling, acid invertase activity is very less and activity of sucrose synthase increases. Sucrose synthase has a central function in the determination of sink strength in storage tissues. In developing seeds of PBG-1, the activity of sucrose synthase increased from 15 to 25 DAF and thereafter a decline was observed till 45 DAF. C. judaicum seeds showed maximum sucrose synthase activity at 15 DAF and then declined till 35 DAF. In PBG-1 seeds, sucrose synthase activity was higher than that of C. judaicum. Kumar and Turner reported significantly higher sucrose synthase enzyme activity in ‘Kaniva’, a large seeded kabuli cultivar than in ‘Sona’, a small seeded desi cultivar. The sink strength of the kabuli chickpea was greater than the sink strength of the desi chickpea, leading to greater accumulation of dry weight in the seeds of the kabuli than desi chickpea. The final seed size was positively correlated with sucrose synthase activity when the seed growth rate was maximal. The present study showed that in developing seeds of PBG-1, sucrose synthase activity higher than that of C. judaicum (Fig. 3) was leading to greater sink strength and seed size in this chickpea cultivar than wild Cicer species. The activity of sucrose synthase in the seeds may, therefore, serve as an indicator of sink strength. The PBG-1 followed the same pattern of enzyme activities as reported in literature that during the early development when mitotic activity is high an invertase mediated pathway of sucrose breakdown operates in seeds. Following the loss of invertase the storage or maturation phase is initiated. Sucrose synthase saves one ATP as compared to invertase and therefore represents an energy saving mechanism of sucrose breakdown. During initiation of storage phase, the concentrations of sugars change from a high hexose levels to high sucrose levels. But C. judaicum followed the opposite pattern, the reducing sugar content was significantly high in mature seeds as compared to that of cultivated variety PBG-1 (Table 2). Acid invertase activity remained higher for a prolonged period in seeds of wild Cicer species and inhibited the transition from rapid cell expansion phase to storage phase and resulting in very small seed size.

The chickpea cultivar PBG-1 seeds exhibited alkaline invertase activity higher than acid invertase activity during active seed filling. Alkaline invertase activity declined with seed maturity. In developing seeds of PBG-1, hexokinase activity was high at 15 DAF and thereafter a decrease in enzyme activity was observed. C. judaicum seeds had maximum hexokinase activity at 25 DAF. PEP carboxylase activity declined subsequently in seeds of C. judaicum resulting in lower activities of this enzyme in developing seeds as compared to the seeds of PBG-1 (Fig. 3). Since legume seeds are rich in proteins, therefore they require a large supply of amino acids for the synthesis of which carbon skeletons are derived from the intermediates of tricarboxylic acid cycle. PEP carboxylase by playing an anapleurotic role in replenishing the intermediates of the above cycle might also be helping the synthesis of amino acids in developing legume

### Table 2—Changes in reducing sugars content (mg/g FW) of chickpea genotypes during seed filling

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<thead>
<tr>
<th>DAF</th>
<th>Podwall</th>
<th>Seeds</th>
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<tr>
<td>C. judaicum</td>
<td>PBG-1</td>
<td>C. judaicum</td>
</tr>
<tr>
<td>15</td>
<td>6.1±0.6</td>
<td>13.4±2.3</td>
</tr>
<tr>
<td>25</td>
<td>4.0±0.1</td>
<td>7.7±0.1</td>
</tr>
<tr>
<td>35</td>
<td>11.0±0.3</td>
<td>1.2±0.3</td>
</tr>
<tr>
<td>45</td>
<td>-</td>
<td>4.8±0.7</td>
</tr>
</tbody>
</table>

maturity at 30 DAF in wild Cicer species. * Differences significant in comparison with PBG-1 at P<0.01; Student’s t-test
seeds. Higher PEP carboxylase contributes a better CO₂ scavenging system in developing seeds of cultivated varieties.

ADPG pyrophosphorylase is the sole enzyme catalyzing the synthesis of the starch precursor molecule, ADP-glucose. ADPG pyrophosphorylase activity increased from 15 DAF towards maturity in *C. judaicum* and PBG-1 seeds, but increase in PBG-1 was 2-3 times higher than that of *C. judaicum* (Fig. 3).

Dejardin *et al.* compared the activities of three
enzymes of the starch biosynthetic pathway (Sucrose synthase, ADPG pyrophosphorylase and starch synthase) at three developmental stages during seed filling (25, 50 and 75% of the dry seed weight) in developing seeds of different pea genotypes. They reported a sharp decrease in the activity of ADPG pyrophosphorylase and starch synthase at 75% dry seed weight (DSW) in Mini and Alaska Sweet, the two wrinkled genotypes of pea. Because of earlier loss of moisture content in C. judaicum seeds, the increase in ADPG pyrophosphorylase was less as compared to PBG-1 seeds.

On the basis of these studies, it can be concluded that high acid invertase activity for a prolonged period in the seeds show a detrimental effect on seed filling and low sucrose synthase activity during storage phase is also an important factor responsible for reduced seed size and biomass of wild species. Higher sucrose synthase in the large-seeded chickpea cultivars particularly during rapid seed filling, appeared to be responsible for greater sink strength in chickpea cultivars than the sink strength of wild Cicer species. As seed size is a major quality determinant in the market, thus, we conclude that increasing sucrose synthase activity than increasing invertase activity is more likely to benefit seed size and considered important in breeding for improved seed size in chickpea irrespective of the growing environment. The biochemical factors i.e. high acid invertase, low sucrose synthase, low ADPG pyrophosphorylase during maturation phase are responsible for lower seed biomass in wild Cicer species. For developing improved chickpea varieties, genes conferring these unfavourable biochemical factors for sink filling may not be transferred from wild species to chickpea cultivars. Only genes contributing stress tolerance, especially that of antioxidant enzymes which provide tolerance against different abiotic stresses need to be transferred to cultivated chickpea. The wild species are rich in expression of many of these antioxidant genes. However, localization of genes beneficial for abiotic stresses and productivity genes need to be worked out, so that only one set of genes especially provide stress tolerance are transferred. Results of present investigation could help in facilitating the exploitation of useful genes from wild unimproved species for improvement of the cultivated species to provide protection against different stresses. Immunity against different stresses will lead to increase in productivity.

References