DOI: 10.1007/s10535-016-0695-2 BIOLOGIA PLANTARUM 61 (2): 359-366, 2017

Differential proline metabolism in vegetative and reproductive tissues determine drought tolerance in chickpea

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Abstract

Proline is emerging as a critical component of drought tolerance and fine tuning of its metabolism under stress affects the plants sensitivity and response to stress. Thus the study was carried out to analyse the effect of water deficit on the proline content and principal enzymes involved in its synthesis (Δ^1-pyrolline-carboxylate synthetase) and catabolism (proline dehydrogenase) at different developmental stages and in different organs (roots, nodules, leaves, pod wall, and seeds) of two chickpea (*Cicer arietinum* L.) cultivars differing in drought tolerance (drought tolerant ICC4958 and drought sensitive ILC3279). It was observed that increased Δ^1-pyrolline-carboxylate synthetase activity under moderate stress in roots and nodules of ICC4958 caused an increase in proline content during initiation of reproductive development whereas increased proline dehydrogenase activity in nodules and leaves at this period helped to maintain reducing power and energy supply in tissues and proper seed development as seed biomass increased consistently up to maturity. On the other hand, roots and nodules of ILC3279 responded to stress by increasing proline content after the developmental phase of reproductive organs was over (near maturity) which negatively affected the response of pod wall to stress. Concurrent increase in activities of Δ^1-pyrolline-carboxylate synthetase and proline dehydrogenase in pod wall of ILC3279 aggravated the oxidative stress and affected seed development as seed biomass initially increased rapidly under stress but was unaffected near maturity.

Additional key words: *Cicer arietinum*, Δ^1-pyrolline-carboxylate synthetase, proline dehydrogenase, water stress.

Introduction

Chickpea is the world second largest cultivated food legume crop with 90 % of its cultivated area in developing countries (Ghosh *et al*. 2015, Millan *et al.* 2015). Terminal drought causes 40 - 50 % reduction in chickpea yield annually (Ahmad *et al.* 2005). Water deficit besides direct effects also causes oxidative stress (Pinheiro and Chaves 2011). Under water stress, major metabolic acclimation mechanism is accumulation of osmolytes like proline, glycine betaine, sugars, *etc*. (Khan *et al.* 2015). Proline provides protection against stress by acting as an osmolyte, N-storage compound, a hydrophilic protectant for enzymes and cellular structures, and as a free radical scavenger (Filippou *et al.* 2013). Proline accumulation in plants observed under stresses might be due to increased proline synthesis or decreased proline degradation (Bagdi and Shah 2013). Besides proline, intermediates of proline metabolism also change during stress, and expression of enzymes involved in different steps of proline metabolism is known to affect the plant stress response. Although proline in plants is synthesised either from glutamate, ornithine, or arginine, the major synthesis pathway activated by osmotic stress uses glutamate as its precursor (Liang *et al.* 2013). For proline synthesis from glutamate, first two steps are catalysed by the rate limiting bifunctional enzyme Δ^1-pyrolline-carboxylate synthetase (PCS), which converts glutamate to glutamic-γ-semialdehyde, which is then converted to proline by Δ^1-pyrolline-carboxylate reductase (PCSR) in the cytosol or chloroplasts (Filippou *et al*. 2013). Rate-limiting step of two-step proline catabolism is catalysed by a flavoprotein proline dehydrogenase (PDH) in the mitochondria. Studying the effect of water deficit stress on enzyme activities involved in proline metabolism in different plant tissues

Submitted 26 November 2015, *last revision* 31 May 2016, *accepted* 2 June 2016.

Abbreviations: DAS - days after sowing, DAF - days after flowering, DM - dry mass, PCS - Δ^1-pyrolline-carboxylate synthetase, PDH - proline dehydrogenase.

Acknowledgements: Davinder Kaur is thankful to the Council of Scientific and Industrial Research, New Delhi for the Junior Research Fellowship to conduct this work.

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can provide valuable information on the physiological significance of its accumulation. Thus, a comparative study was carried out in different tissues, such as roots, nodules, leaves, pod wall, and seeds, of two chickpea cultivars (ICC4958 and ILC3279, drought tolerant and drought sensitive, respectively) under control and water stress conditions during different developmental stages to study the effect of water stress on proline content and key enzymes involved in proline synthesis (PCS) and catabolism (PDH).

Materials and methods

Chickpea (*Cicer arietinum* L.) water stress tolerant cv. ICC4958 and susceptible cv. ILC3279 were sown in randomised block design in the experimental fields of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. Sowing was done in four equal sized plots (three rows of four meters each) and three replicates of each cultivar were sown with a spacing of 30 cm. Crop was irrigated up to 65 d after sowing (DAS). The rain-out shelter was built on two plots at 70 DAS. These plots were neither irrigated afterwards nor received any rainfall indicating water stress conditions. The plots that received irrigation were termed as control. Proline content and proline metabolising enzymes were studied in roots, nodules, leaves, pod wall, and seeds of ICC4958 and ILC3279 under control and water stress conditions at 80 DAS (vegetative growth period), 100 DAS (initiation of reproductive development), and 120 DAS (near maturity). Water stress at 80, 100, and 120 DAS corresponds to mild, moderate, and severe stress, respectively.

Soil samples were collected from deeper (30 - 40 cm below surface) layers of soil in control and water stressed plots, weighed and then water was removed by oven-drying the sample until the constant mass. The moisture content [%] was calculated as [(mass of wet soil - mass of dry soil) / mass of dry soil] × 100.

Pod wall and seeds at different stages of development were taken. Fresh tissue was weighed, then oven dried at 50 °C till a constant mass and their dry mass (DM) was obtained.

Proline content, and Δ^1-pyrolline-carboxylate synthetase (PCS) and proline dehydrogenase (PDH) activities were estimated in roots, nodules, and leaves of ICC4958 and ILC3279 under control and water stress conditions at 80, 100, and 120 DAS. Uniformly developed flowers were tagged. Proline content and proline metabolising enzymes were estimated in pod wall and developing seeds at 7-d intervals during different days after flowering (DAF) till maturity. Proline was extracted from the tissues using 3 % sulphosalicylic acid and estimated by reacting it with acidic ninhydrin reagent (Bates *et al.* 1973).

Tissue samples were homogenized in 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 10 mM mercaptoethanol, 5 mM $MgCl_2$, 0.6 M KCl and 1 % (m/v) polyvinylpyrrolidone in a pre-chilled pestle and mortar. The homogenate was centrifuged at 20 000 *g* and 4 ºC for 30 min. The supernatant was used to assess proline metabolising enzymes. PCS activity was assayed by the method described by Filippou *et al.* (2013). The assay mixture consisted of 100 mM Tris-HCl (pH 7.2), 25 mM $MgCl_2$, 75 mM Na glutamate, 5 mM ATP, 0.4 mM NADPH, and enzyme extract. The reaction velocity was measured as the rate of consumption of NADPH, monitored as the decrease in absorption at 340 nm as a function of time. PDH was assayed following the NAD^+ reduction at 340 nm according to the method of Chen *et al.* (2001). One unit of PDH was defined as 1 nmol(NAD reduced) min^{-1}.

All experimental data recorded were mean values for three replicates with standard deviations (SDs) and statistical analysis was carried out according to the *CPCS-I* package (Cheema and Singh 1993) for factorial completely randomized design (CRD). All data were subjected to analysis of variance (*ANOVA*). Critical differences (CDs) at 5 % level of significance were calculated.

Results

Soil moisture content in control varied depending upon the amount of rainfall received. The control plots received in average 20 mm of rainfall between 80 and 100 DAS and the soil moisture content increased upto 100 DAS (Fig. 1). However, then it decreased back to level at 80 DAS. On the other hand, soil moisture content in plots under rainout shelter decreased continuously creating water stress conditions: at 80 DAS mild stress, at 100 DAS moderate stress, and at 120 DAS severe stress that correspond to 10, 30, and 50 d of exposure to water deficit, respectively (Fig. 1).

Dry mass (DM) of pod wall of ICC4958 under stress was increased by 37 % near maturity as compared to control conditions while DM of pod wall of ILC3279 under stress was decreased on an average by 28 % near maturity (Fig. 2). Seeds of ICC4958 revealed an increase in DM under stress by 2-fold at 21 DAF and by 1.5-fold at 28 and 35 DAF as compared to control conditions. On the other hand, seeds of ILC3279 at 14 DAF revealed a 15-fold increase in DM under stress relative to control (Fig. 2).

Stress initiation decreased the proline content in roots of ICC4958 by 58 % after which it was increased by 33 % at 100 DAS (Fig. 3*A*). On the other hand, in roots of

ILC3279, a 42 % decreased was observed at 100 DAS while at mild and severe stress the proline content increased by 31 and 77 %, respectively. Proline content in nodules of ICC4958 increased by 1.3-fold under stress as compared to control at 100 DAS while in nodules of ILC3279, 6.98 fold higher proline content in nodules under stress was observed at 120 DAS (Fig. 3*A*). Proline content in leaves of ICC4958 was unaffected by mild stress while it increased by 2.95- and 8.2-fold under moderate and severe stress, respectively. On the other hand, proline content in leaves of ILC3279 was 2.1-, 2.3-, and 13-fold higher relative to control under mild, moderate, and severe stress, respectively (Fig. 3*A*).

An average 53 % decrease in proline content was observed during 7 and 21 DAF in pod wall of ICC4958 while 66 % increase was observed in mature pod wall (Fig. 3*B*). On the other hand, a 60, 75, and 74 % increase was observed in pod wall of ILC3279 at 14, 28, and 35 DAF, respectively. Proline content in seeds of ICC4958 under stress decreased by 54 % 35 DAF, while at 14 and 21 DAF, approx. 63 % increase in proline content was observed. Proline content in seeds of ILC3279 was increased on an average by 64 % during their development from 7 to 35 DAF (Fig. 3*B*).

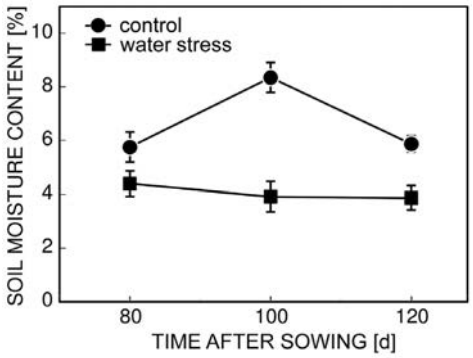

Fig. 1. Soil moisture content profile of control and water deficit stress plots. Means ± SDs, $n = 3$.

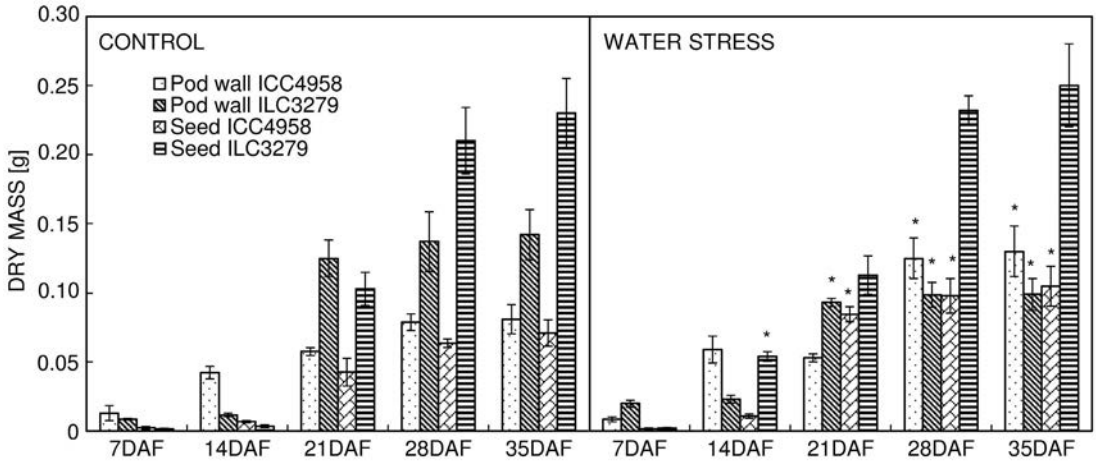

Fig. 2. Effect of water stress on dry mass of pod wall and seeds of chickpea cvs. ICC4958 and ILC3279. Means ± SDs, $n = 3$; * indicate statistically significant differences in comparison to control at $P < 0.05$.

The specific activity of PCS was not much affected in roots of both the cultivars under water stress (Fig. 4*A*). In nodules of ICC4958, specific activity of PCS under mild and moderate stresses was 1.6-fold higher as compared to control while in nodules of ILC3279, PCS specific activity under stress was 1.4- and 3.1-fold less as compared to control at 100 and 120 DAS, respectively (Fig. 4*A*). Initiation of stress decreased the specific activity of PCS in leaves of ICC4958 by 56 % while further stress increased the specific activity by 27 % relative to control. Specific activity of PCS in leaves of ILC3279 under moderate and severe stresses was increased by 63 and 40 %, respectively (Fig. 4*A*).

Pod wall of ICC4958 on an average suffered a 72 % decrease in specific activity of PCS upon stress implication (Fig. 4*B*). On the other hand, specific activity of PCS in pod wall of ILC3279 was increased by 78 and 61 % at 7 and 14 DAF, respectively, while at 35 DAF, 58 % decrease in specific activity was observed (Fig. 4*B*). A 6.4-fold increase in activity was observed in seeds of ICC4958 under stress at 7 DAF while a 6.4-, 19.34-, 15.06-, and 19.57-fold decrease in activity was observed at 14, 21, 28, and 35 DAF, respectively. Specific activity of PCS under stress was 9.7-fold higher in seeds of ILC3279 at 7 DAF after which it decreased and at 35 DAF, it was 1.8-fold lower as compared to control (Fig. 4*B*).

Specific activity of PDH in roots of ICC4958 under water deficit stress was 46 and 88 % lower as compared to control at 100 and 120 DAS, respectively (Fig. 5*A*). Specific activity of PDH in roots of ILC3279 was 42 % higher under moderate stress while severe stress caused a 48 % decrease in specific activity of PDH. Nodules of ICC4958 revealed a 24 and 47 % increase in specific activity of PDH at 80 and 100 DAS, respectively (Fig. 5*A*). No significant effect of water stress was evident on specific activity of PDH in nodules of ILC3279. Enzyme activity in leaves of ICC4958 was

5.9-fold lower at 80 DAS while its 1.6- and 9.1-fold increase was observed at 100 and 120 DAS, respectively. Severe stress caused a 1.7-fold decrease in specific activity of PDH in leaves of ILC3279 (Fig. 5*A*). Pod wall of ICC4958 showed on an average 41 % decrease in PDH activity except at 14 DAF where 39 % increase was observed (Fig. 5*B*). In pod wall of ILC3279, 67, 66, and 23 % increase in specific activity of PDH under stress was observed at 7, 28, and 35 DAF, respectively, while a 54 % decrease was evident at 21 DAF (Fig. 5*B*). Seeds of ICC4958 revealed a 1.7-fold increase in PDH activity under stress at 7 DAF while a 2-fold decrease was evident at 21 and 28 DAF. PDH activity in seeds of ILC3279 under stress was 1.7 fold higher and 1.9 fold lower as compared to control at 21 and 35 DAF, respectively (Fig. 5*B*).

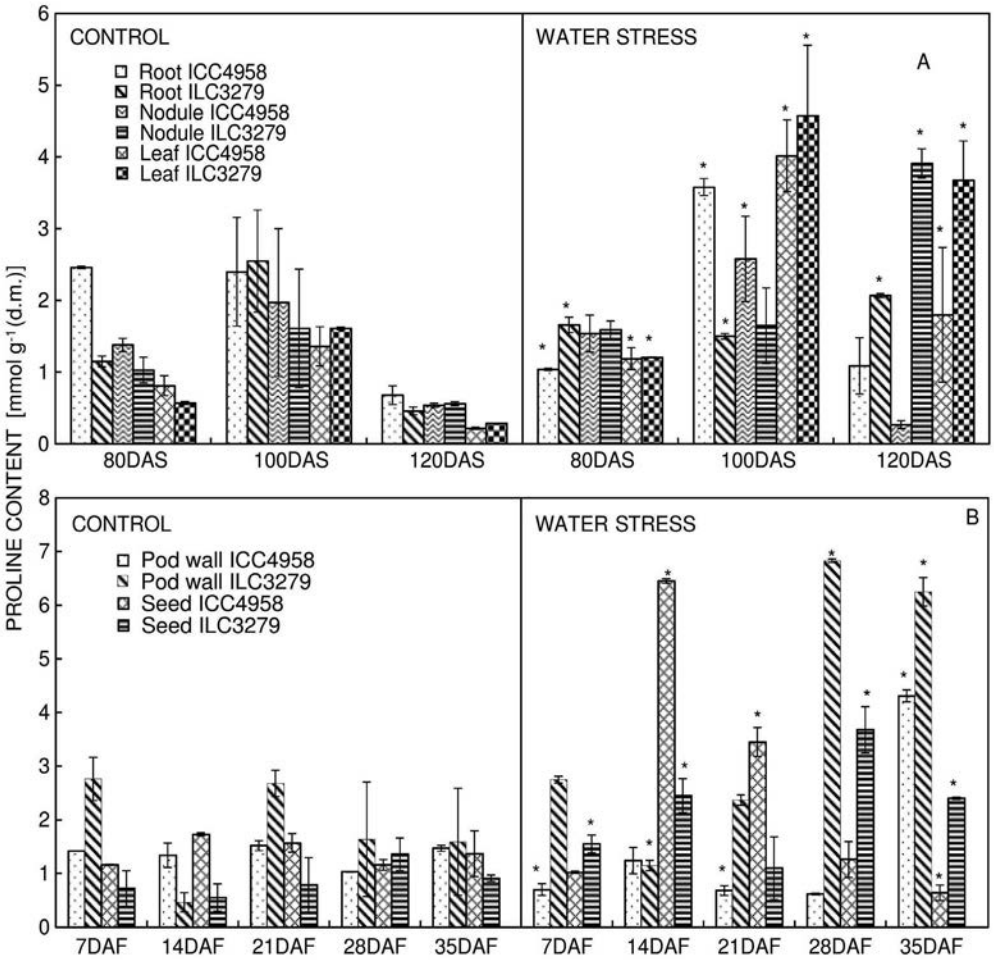

Fig. 3. Effect of water stress on proline content in root, leaf, nodule (*A*), pod wall, and seeds (*B*) of chickpea cvs. ICC4958 and ILC3279. Means ± SDs, $n = 3$; * indicate statistically significant differences in comparison to control at $P < 0.05$.

Discussion

Drought tolerance capacity of a plant is primarily dependent upon a fortified molecular system capable of mitigating stress caused injury (Kato *et al*. 2008). Reproductive phase of chickpea is more sensitive to water stress than vegetative phase (Pushpavalli 2015), thus appropriate water supply to the above ground parts of plant during flower and pod production stage is a pre-requisite to maintain final seed yield (Zaman-Allah 2011). In chickpea, 100 DAS is a critical period for reproductive development when proper water and nitrogen supply is essential for pod wall and seed establishment. In the present study, decreased PDH activity in roots of ICC4958 during this period (Fig. 5*A*) led to an increased proline content (Fig. 3*A*,) which might have helped the tissue to withstand stress as proline is an important osmolyte and antioxidant (Filippou *et al*. 2013) On the other hand, increased PDH activity in roots of ILC3279 caused a decrease in proline content observed in roots of ILC3279 at 100 DAS (Figs. 5*A*, 3*A*). It is apparent that roots of ILC3279 were less efficient in making an efficient time based adaptation to stress. This affected pod wall and seed establishment in ILC3279.

Dehydration is known to induce the expression of the gene encoding for proline carboxylate synthetase (Yoshiba *et al.* 1995) which was probably induced in nodules of ICC4958 and resulted in an increase in PCS activity and proline content (Figs. 4*A*, 3*A*). Earlier studies have reported increase in proline content in plant tissues under a wide array of abiotic stresses such as drought, cold, salinity, *etc*. (Hayat *et al*. 2012). In the present investigation, excess proline in nodules of ICC4958 was catabolised by enhanced activity of PDH (Fig. 5*A*) which is believed to help in maintaining tissue development (Bhaskara *et al.* 2015), generation of ATP *via* supply of reducing power to the mitochondria, feeding Krebs cycle and providing nitrogen from proline (Cecchini *et al.* 2011). On the other hand, stress during initiation phase of reproductive development decreased the specific activity of PCS in nodules of ILC3279 (Fig. 4*A*). Despite decreased PCS activity, proline content in nodules under stress was equal to that under control (Fig. 3*A*) which indicates that proline in the tissue was not generated by *de novo* synthesis but was the result of protein degradation. Therefore, proline in roots and nodules of ILC3279 acted merely as stress marker and thus, stress must have affected water and nitrogen supply to shoot at the time most important to determine the viability of reproductive tissues. In contrast, in ICC4958, no major change was observed in enzymatic activities and proline content in roots and nodules (Figs. 4*A*, 5*A*, 3*A*) when the plants were near maturity which indicates that roots and nodules of ICC4958 attempted to postpone the dehydration of shoot till the seeds get established in the pod so that appropriate water and nitrogen supply to shoot was maintained.

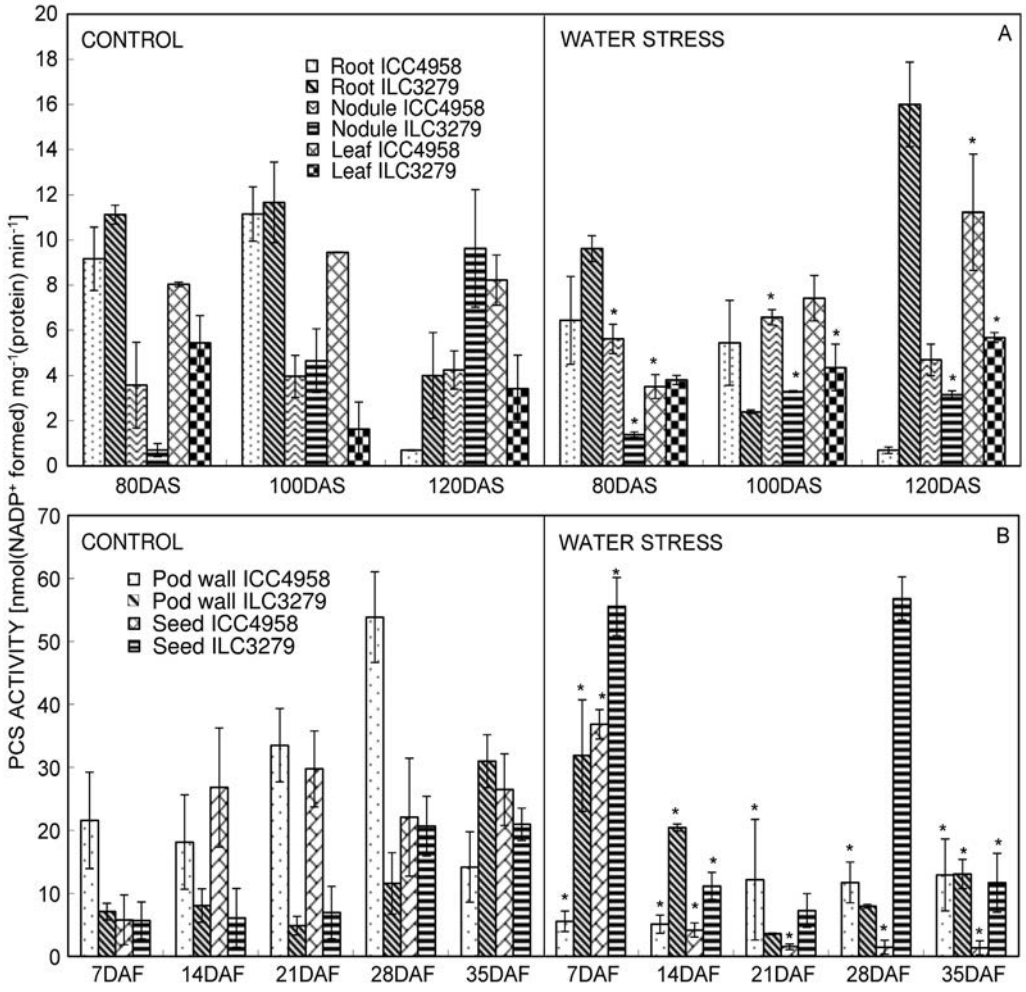

Fig. 4. Effect of water stress on specific activity of Δ^1-pyrolline carboxylate synthetase (PCS) in root, leaf, nodule (*A*), pod wall and seeds (*B*) of chickpea cvs. ICC4958 and ILC3279. Means ± SDs, $n = 3$; * indicate statistically significant differences in comparison to control at $P < 0.05$.

In the present study, increased proline content was observed in leaves of both the cultivars under continued stress exposure, which is believed to help to maintain pressure potential in the tissue and thus alleviate stress caused injury. Similar effect of water deficit stress on proline content in leaves of chickpea has been reported by

Gokmen and Ceyhan (2015). In leaves of ILC3279, increased proline content was the outcome of enhanced PCS and decreased PDH activities (Figs. 4*A*, 5*A*). On the other hand, stress initiation suppressed PDH activity in leaves of ICC4958, but the activity at 100 DAS was increased (Fig. 5*A*), probably to supply energy and nutrients to the tissue required for stress recovery (Cecchini *et al.* 2011). Transaminases, such as glutamate: pyruvate aminotransferase, get activated under stress when carbon shortage becomes a limiting factor for proper metabolism and use of amino acids, such as glutamate, as a carbon source to feed the Krebs cycle (Miflin and Habash 2002). In our study, increased PDH activity in leaves of ICC4958 might have contributed to channel excess proline to glutamate. Moreover, glutamate is a known precursor of chlorophyll synthesis in leaves (Forde and Lea 2007). Thus, it can be proposed that in leaves of ICC4958, excess proline was utilized to sustain Krebs cycle and for chlorophyll synthesis and thus helped to maintain photosynthetic capacity under stress. From these observations, it is evident that leaves of both the cultivars increased proline content, thus, maintaining osmotic environment, but leaves of ICC4958 possessed better adaptive features under stress which might have assisted in maintaining appropriate photoassimilate supply to the pod wall.

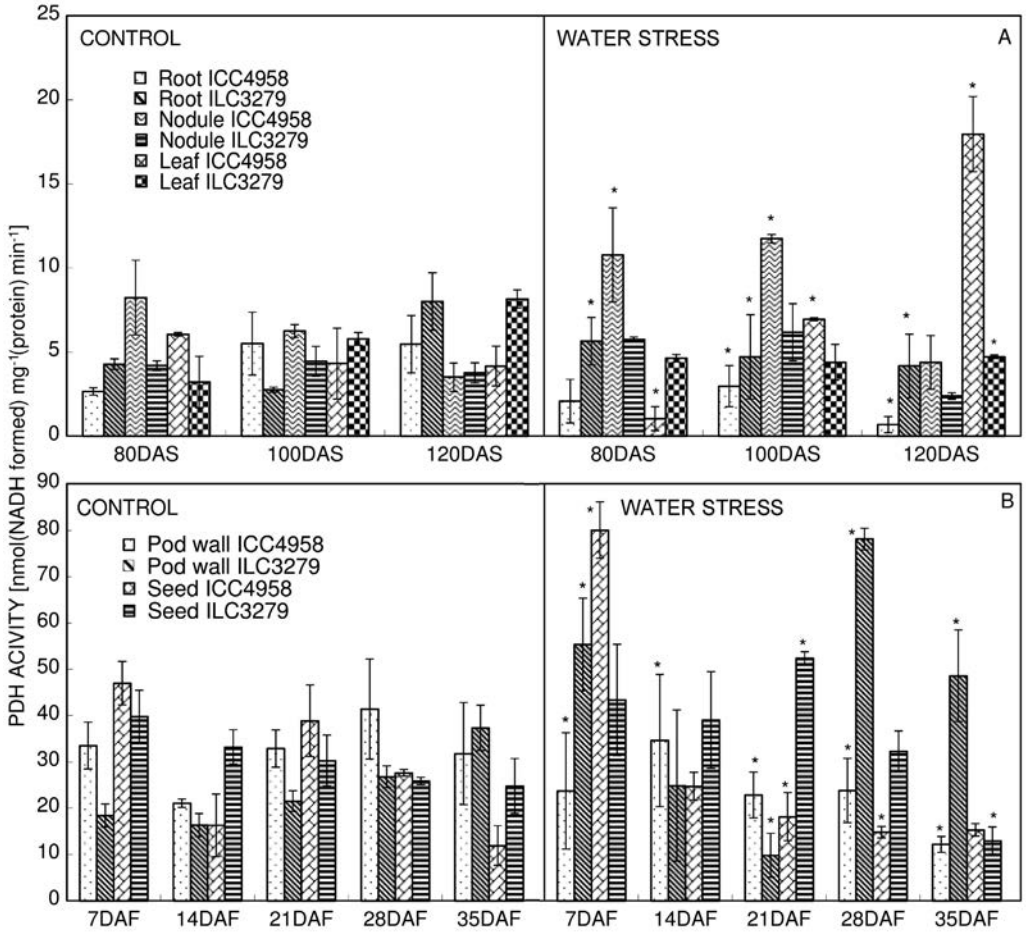

Fig. 5. Effect of water stress on specific activity of proline dehydrogenase (PDH) in root, leaf, nodule (*A*), pod wall, and seeds (*B*) of chickpea cvs. ICC4958 and ILC3279. Means ± SDs, $n = 3$; * indicate statistically significant differences in comparison to control at $P < 0.05$.

Decreased PCS activity in pod wall of ICC4958 led to decreased proline content during early developmental stages which indicates less intensity of stress experienced by pod wall because stress was largely alleviated by roots, nodules, and leaves (Figs. 3*B*, 4*B*). The PDH activity in young pod wall of ICC4958 was also reduced (Fig. 5*B*) which can be an adaptive mechanism to avoid overproduction of glutamic acid in young tissue because glutamic acid is metabolised to γ-aminobutyric acid (GABA) *via* GABA shunt and GABA is known to down-regulate the genes associated with cell-wall modifications (Batushansky *et al.* 2014) required for developmental changes (Michaeli and Fromm 2015). Thus, for proper development of pod wall, PDH activity was reduced during early developmental stages. Seeds at young developmental stages need to equip themselves with

enhanced defence system to avoid early seed death. Thus, seeds of ICC4958 during early developmental stages possessed increased proline content to avoid dehydration in the tissue (Fig. 3*B*). Dry mass of seeds of ICC4958 was unaltered during early growth stages while it increased near maturity, which indicates rational translocation of assimilates by pod wall, which acted as a source for the seeds (Fig. *2*). This might also help to enhance the quality of seeds as seed size of chickpea is a critical factor which determines subsequent plant growth parameters like germination, seedling vigour, *etc*. (Gul *et al*. 2015). After the seed establishment in the pod of ICC4958 was successful, lower stress in the seeds of ICC4958 near maturity was marked by decrease in proline content. On the other hand, increased PCS activity under stress in pod wall and seeds of ILC3279 caused an increase in proline content. The increase in free proline content in sensitive cultivars under water deficit stress is often correlated with protein degradation (Glaubitz *et al*. 2015). In the present study, a concurrent increase in activities of PCS and PDH in pod wall of ILC3279 at early developmental stages might have caused increased content of reactive oxygen species *via* proline-P5C cycle (Qamar *et al.* 2015), thus aggravating the stress condition. Thus, young pod wall of ILC3279 suffered oxidative damage which resulted in significant reduction in DM near maturity. Moreover, increased DM of seeds during early stages of development (Fig. 2) might have caused wastage of sink as rapid development of storage organs like seed is known to cause source limitation (Gan *et al*. 2004). From our study, it can be concluded that efficient temporally based adaptation to stress by roots and nodules and rational refinement of metabolism in the leaves, pod wall and seeds of ICC4958 are the main factors contributing to its water stress tolerance as compared to ILC3279 (Fig. 6). Thus roots and nodules of ICC4958 possess better potential to sustain water and nitrogen supply to leaves, pod wall, and seeds during initiation of reproductive development and leaves, pod wall, and seeds modify proline metabolism in favour of successful pod wall and seed set. On the other hand, in ILC3279, a lack of synchronisation between the onset of adaptive mechanisms in roots and nodules and pod wall and seed initiation along with rapid alterations in proline metabolism in leaves, pod wall, and seeds was observed (Fig. 6). The study provides novel information on improving our understanding about proline metabolism in chickpea plants subjected to water stress.

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