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Changes in protein pattern during different developmental stages of somatic embryos in chickpea

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Abstract

Mature embryonic axes were used for chickpea (*Cicer arietinum* L.) regeneration *via* somatic embryogenesis. Qualitative and quantitative estimation of protein profile during somatic embryogenesis by SDS-PAGE and densitometric analysis showed differential expression of various storage proteins at different stages of somatic embryo development, which was compared with the profile of developing seeds. Total protein content in somatic embryos of chickpea increased from globular stage [2.9 μg mg^{-1}(f.m.)] to cotyledonary stage [4.8 μg mg^{-1}(f.m.)] and then started decreasing during onset of maturation and germination [up to 1.5 μg mg^{-1}(f.m.)]. Differential expression of seed storage proteins, late embryogenesis abundant (LEA) proteins and proteins related with stress response were documented at different stages of somatic embryogenesis. Germinating somatic embryos showed degradation products of several seed storage proteins and the appearance of new polypeptides (76.8, 67.6, 49.9 and 34.2 kDa), which were absent during differentiation of somatic embryos. A low molecular mass (17.7 kDa) polypeptide was uniformly present during all stages of somatic embryogenesis and it may belong to a group of stress-related proteins. This study describes the expression of true seed storage proteins like legumin, vicilin, convicilin and their subunits at different stages of somatic embryogenesis, which may serve as excellent markers for embryogenic pathway of regeneration in chickpea.

Additional key words: *Cicer arietinum*, mature embryonic axes, SDS-PAGE, seed storage proteins.

Introduction

Somatic embryogenesis is the process where somatic cells are differentiated into embryogenic state, which go through a series of morphogenetic and biochemical changes resulting in the formation of somatic embryos. The process of somatic embryogenesis where fusion of gametes is not involved differs from zygotic embryo development (Jimenez 2001). The somatic embryogenesis can be used as a model system for the study of cellular differentiation, physiological, molecular and biochemical events occurring during the onset and development of embryos in higher plants. Biochemical characteristics of the first induction phase of somatic embryogenesis have been so far investigated at protein level in many plant species while studies during the second expression phase are restricted to few plants (Tchorabadjieva 2005). Somatic embryogenesis is characterized with the appearance or disappearance of a number of specific proteins and enzymes during the course of differentiation and such proteins have been identified as molecular markers.

It appears that accumulation of proteins at maturation step is crucial for successful conversion of somatic embryo into plantlets (Lai and McKersie 1994). Expression of seed storage proteins, particularly globulins, showed spatial and temporal expression during the course of somatic embryo development. Differential expression of true seed storage proteins during embryogenesis has been reported in several legumes (Domoney

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Abbreviations: BA - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; DAP - days after pollination; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; LEA - late embryogenesis abundant; MEA - mature embryonic axis; PEG - polyethylene glycol; SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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et al. 1980, Shewry and Casey 1999). In addition to storage proteins, several other proteins like lectins, proteinase/amylase inhibitors, late embryogenesis abundant (LEA) proteins are also synthesized in the somatic embryos. Apart from possibly serving as storage proteins, these proteins have also been implicated as defense proteins against abiotic stresses (He and Fu 1996, Dita *et al*. 2006). The accumulation of storage products in somatic embryos exhibits similar characteristics as in the zygotic embryos (Merkle *et al*. 1995). However, the amount of a particular storage and LEA proteins as well as the timing of their accumulation may differ between somatic and zygotic embryos, which are usually regulated by abscisic acid (ABA) and or water stress induced gene expression (Merkle *et al*. 1995, Yeung 1995). Such differential accumulation of proteins may provide important clues to the function and regulation of genes associated with embryogenesis. As concerning legumes, proteins related with somatic embryogenesis have been studied in alfalfa (Stuart *et al*. 1988), soybean (Stejskal and Griga 1995), peanut (Roja-Rani *et al*. 2005), pea (Griga *et al*. 2007) and chickpea (Ghanti *et al*. 2009).

Complete plant regeneration *via* embryogenic mode has been reported in chickpea from immature cotyledonary segments, young leaflet and mature embryonic axes (Sagare *et al*. 1993, Kumar *et al*. 1994, Ghanti *et al*. 2010). However, information on biochemical changes during embryo-genesis in chickpea is inadequate. The relationship between somatic embryogenesis from leaf explant in relation to ethylene and methane ratio was studied in four cultivars of chickpea (Guru *et al*. 1999). Recently, Ghanti *et al*. (2009) has reported the role of various enzymes related with oxidative burst like peroxidase, catalase, phenyl-alanine ammonia lyase and malate dehydrogenase, during different developmental stages of somatic embryogenesis and monitored the several stage specific protein bands in chickpea somatic embryo. However, to our knowledge, no previous reports are available in chickpea for investigation of qualitative and quantitative protein pattern associated with somatic embryogenesis and their comparison with zygotic embryogenesis. The objective of the current work was to analyze the differential expression pattern of seed storage and other proteins during development of chickpea somatic embryos and its comparison with the developing seeds.

Materials and methods

Four chickpea (*Cicer arietinum* L.) cultivars (P362, P372, C235 and JG16) were used in initial experiments for the isolation of total protein from mature embryonic axes (MEA) and derived calli, whereas leaf explant was used as a negative control. Detailed analysis of different developmental stages of somatic embryos was done only with cv. P362, mainly because of its good production of somatic embryos of particular developmental stage, which was necessary for sampling. Seeds of chickpea were soaked in 1 % *Teepol* for 10 min. After 3 - 4 rinses with sterile water *Milli-Q* (*Millipore*, Bedford, USA; MQ), they were surface sterilized in 0.1 % $HgCl_2$ (m/v) for 5 min followed by 3 - 4 washings with sterile MQ water. The seeds were immersed in 70 % ethanol for 2 to 3 min and after washing with sterile MQ water, soaked in water. Mature embryonic axes were isolated from overnight soaked seeds, 2 mm of epicotyl and 2 - 3 mm hypocotyl were removed for preparing explants (decapitated MEA). Callus was induced from excised mature embryonic axes on Murashige and Skoog (1962; MS) basal medium supplemented with B5 vitamins (Gamborg *et al*. 1968), 3 % (m/v) sucrose, 0.01 % myo-inositol, 0.8 % (m/v) agar and 5 mg dm^{-3} 2,4-dichloro-phenoxyacetic acid (2,4-D) for 10 d and then sub-cultured on the same medium with reduced auxin concentration to 0.05 mg dm^{-3} and supplemented with 200 mg dm^{-3} casein acid hydrolysate for 5 d and then sub-cultured on the same medium without auxin, for differentiation. Embryogenic calli with globular and heart shaped embryos were transferred to maturation medium consisting of MS basal medium supplemented with 1.0 mg dm^{-3} 6-benzylaminopurine (BA), 3.0 mg dm^{-3} gibberellic acid (GA_3), 0.02 mg dm^{-3} indole-3-acetic acid (IAA) and 7.5 % (m/v) polyethylene glycol (PEG). The pH of the medium was adjusted to 5.8 prior to autoclaving. Morphologically well characterized somatic embryos of different developmental stages such as globular, heart-shaped, torpedo, cotyledonary and germinating stage and leaf tissue, mature embryonic axes and calli derived from them were collected for analysis. The samples were frozen in liquid nitrogen and stored at -80 °C before analysis. Different developmental stages of chickpea seeds were also collected from field grown plants, 2 d after pollination till maturation of the seed.

The samples of somatic embryos (10 mg) and developing seeds (100 mg) were ground in a pre-chilled mortar and pestle in the ratio of 1:10 in extraction buffer. The protein content of somatic embryos and developing seeds was determined according to Bradford (1976). Samples were homogenized with extraction buffer containing 50 mM Tris-HCl (pH 7.5), 200 mM sodium chloride, 2.5 mM phenylmethylsulphonyl fluoride, 1 mM ethylenediamine tetraacetic acid, 14 mM β-mercapto-ethanol and incubated on ice for 30 min and then centrifuged at 4 °C for 20 min at 14 000 *g*. The super-natant was collected and samples were used for protein quantitation and analysis. The amount of protein was calculated using the calibration curve, prepared with a solution of bovine serum albumin (BSA). Three replicates of each stage were independently used for

protein extraction. Data were statistically analyzed using *SPSS* software package *v. 13.0* (*SPSS Inc*., Chicago, USA), using Duncan's multiple range test (DMRT).

Electrophoretic analysis of somatic embryos as well as developing seeds was carried out according to Laemmli (1970). The protein samples were mixed with sample buffer consisting of 0.5 M Tris-HCl (pH 6.8), 10 % glycerol, 4 % β-mercaptoethanol, 2 % SDS and bromophenol blue as the tracking dye. The preparations were boiled at 98 °C for 3 min and proteins were separated using discontinuous SDS-PAGE (12 % running gel pH 8.8 and 5 % stacking gel pH 6.8) at room temperature in Tris-glycine buffer (pH 8.3). Electrophoretic separation of cell free extracts on 12 % denaturing SDS-PAGE was performed at constant current of 25 mA. Medium range molecular mass marker (*Bangalore Genei*, Bangalore, India) was used as standard. After electrophoresis, the gels were stained with either 0.5 % Commassie brilliant blue 250 or 0.2 % silver nitrate (Sambrook and Russell 2001). Silver staining of gels was performed first by fixation in 30 % ethylalcohol and 0.5 % acetic acid solution, sensitization with 0.02 % sodium thiosulphate followed by treatment with 0.2 % silver nitrate solution and finally developing in solution containing 37 % formaldehyde, 3 % potassium carbonate and 0.001 % sodium thiosulphate. The gels were photographed and analyzed by using *Quantity One*, 1-D analysis software (*Bio-Rad*, Hercules, USA).

Results

In MEA, the quantity of total soluble protein (TSP) was from 37.5 to 47.4 μg mg^{-1}(f.m.) in all four cultivars (P362, P372, C235 and JG16) tested. In calli derived from MEA, the protein content decreased more than 10 times as compared to the initial explant and were 4.23 to 4.69 μg mg^{-1}(f.m.). This could be mostly due to high relative water content in calli (data not shown). The protein content of somatic embryos increased from globular stage (2.9 μg mg^{-1}) up to cotyledonary stage (4.8 μg mg^{-1}) and then decreased during onset of maturation and germination to 1.54 μg mg^{-1} (Table 1). Protein content in developing chickpea seeds increased from 2.92 μg mg^{-1} (2 DAP) to 72 ± 5 μg mg^{-1} (48 d; Table 1). The germinating mature seeds during the early stages (2 - 20 h) showed significant decrease in protein content in zygotic embryonic axes from 42 ± 4 to 15.12 ± 2 μg mg^{-1} and after 24 - 30 h of germination it showed increasing trend with increase in growth. Similarly, the cotyledonary stage somatic embryos during *in vitro* germination showed decrease in protein content to 58 - 62 % of initial value and subsequently reflected increasing pattern with growth of juvenile plantlets (Table 1).

Results of SDS-PAGE (Fig. 1) and densitometric analysis (data not shown) showed differential protein expression pattern during the different developmental stages of somatic embryogenesis (Fig. 2). The key storage proteins of chickpea seed vicilin major subunit (48 - 50 kDa), vicilin minor subunit (25 - 28, 30, 34 - 37 kDa), convicilin (68 - 72 kDa), legumin α (40 - 44 kDa) and legumin β (20 kDa) appeared at different stages of somatic embryos in chickpea (Fig. 2*A*,*B*.). Expression of key storage proteins in somatic embryos did not show a dramatic increase from globular to cotyledonary stage.

Table 1. Protein content [mg g^{-1}(f.m.)] in developing embryos at different stages of somatic and zygotic embryogenesis. Means ± SE of three independent experiments.

	2 d	6 d	12 d	16 d	22 d	28 d	32 d	38 d	42 d	48 d
Somatic	2.90±0.12	3.88±0.18	4.18±0.24	4.88±0.20	5.66±0.18	5.42±0.16	1.54±0.18	2.82±0.14	5.22±0.14	6.10±0.28
Zygotic	2.92±0.32	7.25±0.24	7.38±0.38	7.94±0.30	25.56±0.62	30.96±0.34	42.72±0.28	58.88±0.28	62.74±0.28	72.32±0.28

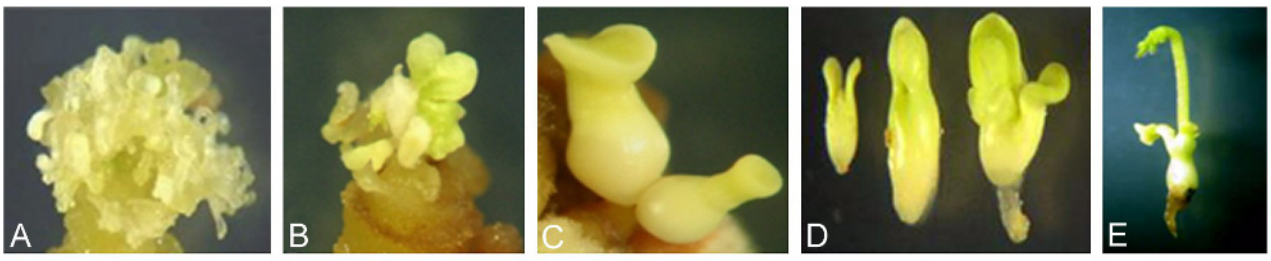

Fig. 1. Different stages of chickpea somatic embryo: *A* - globular stage (15 - 22 d), *B* - heart-shaped stage (22 - 32 d), *C* - torpedo stage (32 - 42 d), *D* - cotyledonary stage (38 - 48 d), *E* - germinating stage (> 42 d).

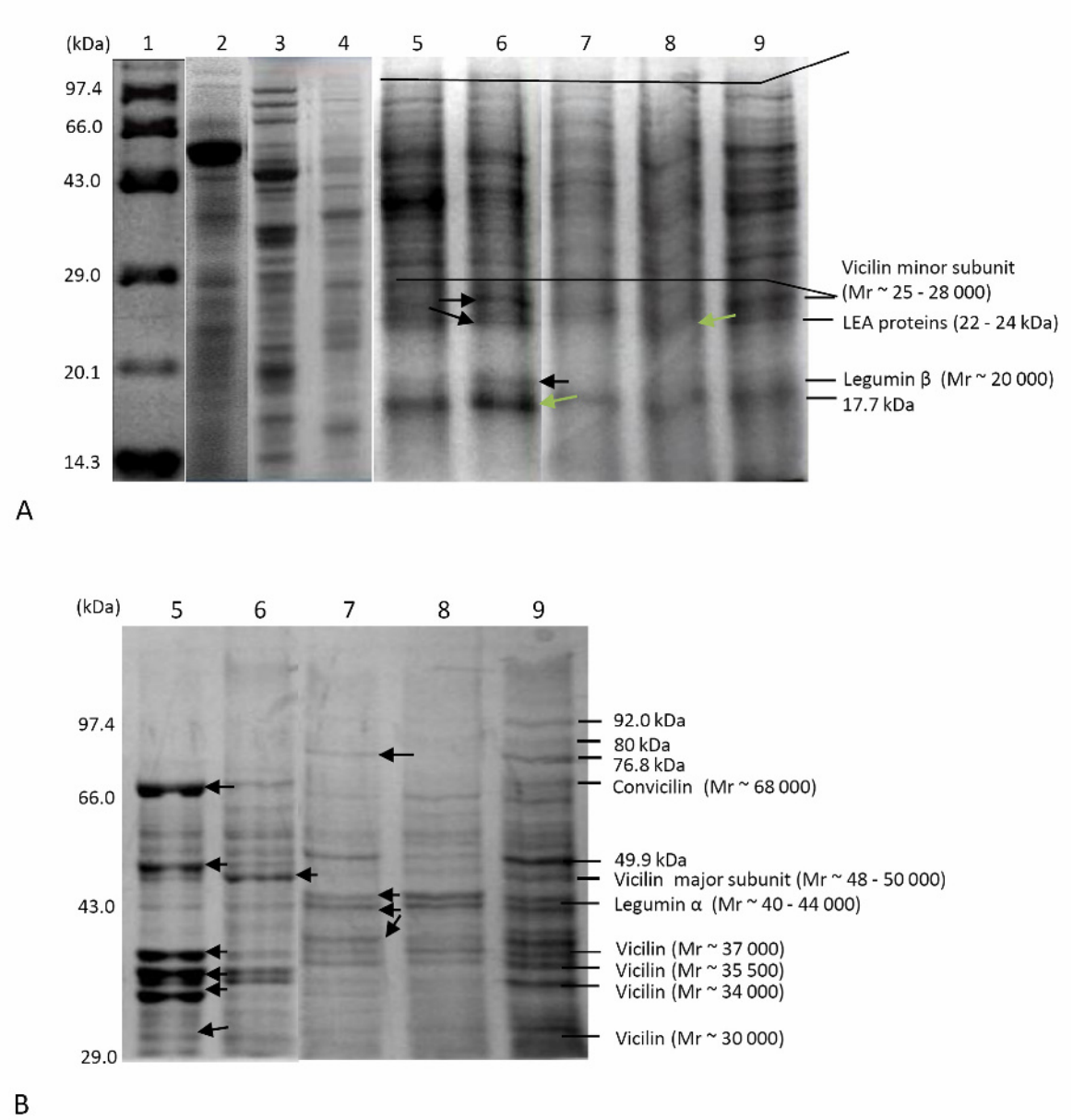

Fig. 2. Protein patterns (SDS-PAGE) of different stages of somatic embryos. *A*: *lane* 1 - molecular mass markers, 2 - leaf, 3 - mature embryonic axes, 4 - calli derived form mature embryonic axes, 5 - globular, 6 - heart, 7 - torpedo, 8 - cotyledonary, 9 - germinating stage. *B*: enlarged view of a portion of SDS-PAGE (*A*). *Black arrows* indicate the position of key storage proteins and corresponding molecular mass of important protein bands (Mr). Portion of gel 2*A* enclosed within the two lines is enlarged to reveal the finer details in 2*B*.

Globular stage somatic embryos expressed comparable, even higher content of vicilin major subunit, minor subunit and convicilin, as compared to more advanced developmental stages. Vicilin minor subunit (28 kDa) was maximally expressed during heart stage. Legumin α was mainly observed in torpedo and cotyledonary stages with maximum expression in the cotyledonary embryo. Vicilin minor subunit (30 kDa) and a low molecular mass polypeptide (17.7 kDa) were present at every stage of somatic embryo development. Germinating embryos exhibited not only storage proteins like vicilin, convicilin, legumin degradation products, but also showed intense expression of 76.8, 67.6, 49.9 and 34.2 kDa polypeptides, which were absent during the other developmental stages of somatic embryos. The 92 and 80 kDa polypeptides mainly appeared during torpedo stage embryos. The storage protein legumin β was mainly present during the globular and heart stage embryos. Late embryogenesis abundant (LEA) proteins of 22 - 24 kDa were present only at detectable level in cotyledonary stage somatic embryos. Expression of these seed-specific storage proteins were completely absent in somatic tissues of leaf and showed a completely altered protein pattern (Fig. 2*A*).

Developing chickpea seed showed temporal and

spatial accumulation of typical seed storage proteins (Fig. 3). Lipoxygenase (96.4 kDa) appeared at late stages of seed development with maximum expression in mature seed. Convicilin (68 - 70 kDa) was maximally expressed in early stages (2 - 8 DAP) of seed development and expression of 62 kDa polypeptide started to increase from 2 to 10 DAP and remained stagnant for 14 DAP, then started to decrease. It is interesting, that early stages of seed development (2 - 6 DAP) exhibited higher expression of vicilin subunit than developmentally advanced stages of chickpea seed. The legumin α was expressed after 10 DAP and showed maximum expression at mid-maturation stage (14 - 22 DAP) and then started to decrease. Vicilin subunit of 30 and 34 - 37 appeared from early stages of seed development and showed consistent expression up to mid-maturation stage and started to disappear in the later stages of seed development (22 to 36 DAP). Vicilin minor subunit band started to appear in the later stages of seed development. The 28.0 kDa polypeptide was consistently expressed during all the developmental stages of chickpea seed. LEA proteins of 22 - 24 kDa appeared maximally during the later stages of seed development. Expression of β-lectin (< 20 kDa) was maximum during the later stages (24 - 32 DAP) of seed development (Fig. 3).

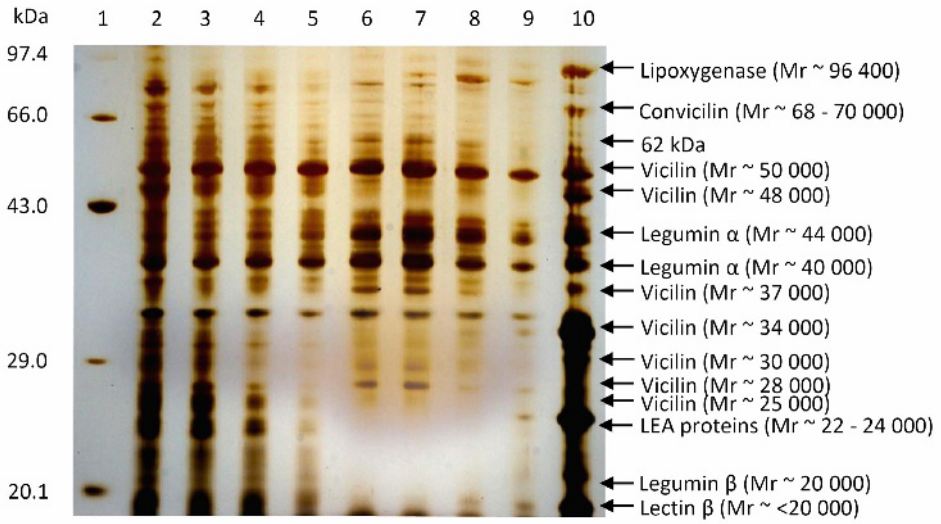

Fig. 3. Protein patterns (SDS-PAGE) of different developing stages of chickpea seed. *Lanes* 1 - molecular mass marker, 2 - seed 2 d after pollination (DAP), 3 - 6 DAP, 4 - 10 DAP, 5 - 14 DAP, 6 - 18 DAP, 7 - 22 DAP, 8 - 26 DAP, 9 - 32 DAP, 10 - mature seed. *Black arrows* mark the position of key storage proteins with corresponding molecular mass (Mr) on the *right side* of gel.

Discussion

Most of the grain legumes are recalcitrant to *in vitro* culture, which hampers the development of reliable regeneration techniques. Better understanding of the fundamental processes that trigger and control somatic embryogenesis will lead to more rational regeneration protocols and their further applications (Lakshmanan and Taji 2000). The characterization and functional analysis of protein markers for somatic embryogenesis, offers the possibility of determining the embryogenic potential of plant cells in culture much before any morphological changes have taken place and of gaining further information on the molecular basis of induction and differentiation of plant cells (Tchorabadjieva 2005). The most significant progress in understanding the molecular and cellular events of early embryogenesis has resulted from experiments on somatic embryos of a few plant species (Zimmerman 1993). Qualitative and quantitative turnover of proteins during different stages of differentiation seems to play an important role for recovery of mature well developed somatic embryos in legumes (Griga *et al*. 2007). In present study, we have measured protein content and analyzed the protein pattern during different stages of somatic embryogenesis in chickpea as well as in developing seeds.

The critical stage in somatic embryo development is the phase of maturation, marked by a period of cell expansion and reserve accumulation following differentiation, which determines the successful germination and regeneration of plantlets. Various physiological and biochemical events associated with reserve accumulation and mobilization in somatic embryos showed remarkable similarities and differences with those events during zygotic embryogenesis. Accumulation of proteins in chickpea somatic embryos increased from globular stage to maximum in cotyledonary stage, followed by decrease during germination stage, which is similar to alfalfa,

soybean and pea (Lai *et al*. 1992, Stejskal and Griga 1995, Griga *et al.* 2007). However, there was a slight variation in total protein content observed for torpedo and cotyledonary stage embryos because in chickpea somatic embryos (as well as in another leguminous species), the cotyledons do not represent major part of fully morphologically developed individual and it is represented by robust hypocotyl only (Krochko *et al*. 1992). Morphologically and biochemically, the most dramatic events of seed development also occur during maturation. These include rapid expansion of the cotyledons and concomitant synthesis and accumulation of proteins, lipids and in some cases sugars and ABA in the seed to ensure embryo dormancy.

The biosynthesis of key storage proteins of chickpea seed like vicilin major subunit, vicilin minor subunit, convicilin, legumin α and legumin β was apparent during different stages of somatic embryos as reported earlier (Vitale and Bollini 1995). The globular and heart stage somatic embryos, which are like early stages of developing seeds have shown comparable or even higher expression of vicilin subunits and, whereas other key storage proteins like α-legumin, that are expressed during late stages of seed development, appeared during torpedo and cotyledonary stages of somatic embryos. The expression of storage proteins in chickpea somatic embryos was relatively low as compared to zygotic embryos of mature dry seed, that is similar to earlier reports in alfalfa (Stuart *et al*. 1988), soybean (Dahmer *et al*. 1992) and pea (Griga *et al*. 2007). Lipoxygenase, which is mainly located in storage parenchymatous tissues of cotyledons, was absent in all the developing stages of somatic embryos. LEA proteins of 22 - 24 kDa were present only in detectable levels in the cotyledonary stage somatic embryos. The differences observed in seed storage protein pattern between somatic embryos and developing seeds could be attributed to differential accumulation of storage proteins during maturation stages and differences in the surrounding environment of *in vitro* developing somatic embryo compared to seed development *in planta* (Griga 1999). Absence of endosperm, testa and true cotyledons in somatic embryos are the major morphological and physiological differences from the zygotic embryos. Low molecular mass polypeptide of 17.7 kDa was present in all the stages of somatic embryogenesis. This protein may belong to a group of heat-shock proteins or protein related to stress response and seems to play a role in differentiation of pro-embryogenic mass into somatic embryo (He and Fu 1996). Recently, the variability of high and low molecular mass glutenin subunits have been used for the evaluation of genetic diversity in Spanish rivet wheat (Carmona *et al*. 2010). In our study, the expression of different seed storage proteins and other associated proteins during developing somatic embryos can be used as a marker to assess the extent of optimum development and maturation potential of somatic embryos for developing new plantlets. Further characterization of these proteins of chickpea shall be extended to identify the specific protein that triggers the pathway of somatic embryogenesis in recalcitrant grain legume chickpea.

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