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Full Length Research Paper

Changes in protein composition and protein phosphorylation during somatic embryogenesis and plant regeneration in peanut (*Arachis hypogaea* L.)

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Changes in protein profiles and protein phosphorylation were studied in various stages of germinating somatic and zygotic embryos. Many proteins, which were expressed in cotyledonary stage somatic embryos, were also present in the zygotic embryos obtained from mature dry seed. The intensity of 22 kDa protein was much higher in zygotic embryos in comparison to cotyledonary somatic embryos. Proteins of 55, 53, 27 and 25 kDa did not vary in expression during different stages of germination of somatic and zygotic embryos. There was rapid accumulation of 28 kda protein in germinating zygotic and somatic embryos at 3rd stage and persisted during the subsequent stages of germination indicating the emergence of radical and plumule. A clear difference was observed in the patterns of protein phosphorylation in germinating somatic and zygotic embryos. A 50 kDa protein was heavily phosphorylated in mature zygotic embryos at 2nd stage of germination and disappeared in 3rd stage of germination. Protiens of 50 and 43 kDa proteins showed enhanced phosphorylation up to 3rd stage and drastic reduction was observed at 4th stage. In addition, phosphorylation of 68 and 65 kDa specifically appeared in 3rd stage of germinating somatic embryos and were not observed during any germinating stages of zygotic embryos.

Key words: Protein analysis, sodium dodecyl sulphate polyacrylamide gel electrophoresis, protein phosphorylation, zygotic embryo axis.

INTRODUCTION

Groundnut or peanut (***Arachis hypogaea***) is one of the principle edible oil seed and protein rich leguminous crop, cultivated on over 20 million hectors in over 108 tropical and subtropical countries, with an annual yield of seeds estimated 28 million tons (Fao, 2007). Peanut is high oil yielding and protein rich legume. Current commercial peanut cultivars are allotetraploids, apparently derived from a single hybridization event between diploid peanut, ***A. hypogea*** and ***Arachis ipaensis*** (Kocher, 1996). Conventional methods of plant improvement have met with limited success in development of the disease resistant cultivars due to narrow genetic variability present in the groundnut germplasm. Such variability has become a barrier in crop improvement of cultivated species due to frequent failures in interspecific crosses such as post fertilization barriers leading to abortion of embryos, seed or pods, which fail to germinate in the soil (Johanson and Smith, 1956). The crop has narrow germplasm base without satisfactory resistant to major pathogens and viruses. The development of suitable protocol for genetic improvement of the plants using biotechnological methods is the pre-requisite (Rey et al., 2000).

Application of tissue culture for the improvement has been limited due to lack of useful methods to recover plants from tissues and cells culture ***in vitro*** (Mroginski et al., 1981; Ozais-akins, 1989; Baker and Weitstein, 1992).

Comparatively with respect to plant transformation, plant regeneration through somatic embryogenesis and organogenesis may be useful. Somatic embryogenesis

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has been reported from immature embryo axis (Eapen and George, 1993; Baker et al., 1994), mature embryos (Chengalrayan et al., 1994; Baker et al., 1995), mature embryo derived leaflets (Chengalrayan et al., 1997; Venkatachalam, 1996), mature and immature cotyledons (Gill and Saxena, 1992; Durham and Parrott, 1992; Wetstein and Baker, 1993), leaflet cultures (McKently 1991; Gill and Saxena, 1992; Baker and Wetzstein, 1998), hypocotyls (Venkatachalam et al., 1997) and epicotyls (Little et al., 2000). Although protocols for the regeneration of peanut ***via*** somatic embryogenesis and organogenesis have been developed, most of them resulted in low frequencies of plant recovery ranging from 18-38% (Ozais-Akins, 1989; Reddy and Reddy, 1993; Wetzstein and Baker, 1993).

Few reports have described methods for obtaining high frequency plant conversion from somatic embryos. Little et al. (2000) reported that a combination of genotype VC1 and picloram at 83.0 µM resulted in the best conversion efficiency of somatic embryos (59.5%) and also the highest average number of mature somatic embryos/mg of tissue. A reliable protocol of plant regeneration for efficient application of genetic transformation has been published by us in which we obtained 80% of shoot induction from somatic embryos obtained from somatic embryos isolated from 60 day old cultures, and shoots rooted with frequency of 93.3% of plant recovery (Roja and Padmaja, 2005), but actual reason for lack of hundred percent plant recovery is yet to be known.

There is no report available in groundnut on compareson of somatic and zygotic embryogenesis at the biochemical and molecular levels. Identification of biochemical and molecular markers characteristic to different stages of somatic embryogenesis, in comparison with zygotic embryos would be helpful in improving the regeneration potential of somatic embryos.

MATERIALS AND METHODS

Samples of different germinating stages of somatic and zygotic embryos

Zygotic embryo axes were excised from mature dry seed were sterilize in 70% alcohol for 1 min followed by 0.1% $HgCl_2$ for 20 min and thoroughly rinsed three times in sterile distilled water and inoculated on MS basal medium for germination. The samples at different germinating stages of zygotic embryo axes were collected within 8-9 days for extraction of protein.

The pods collected from the field grown plants were washed in liquid detergent (teepol) solution (5%, v/v) for 15 min and rinsed thoroughly in distilled water. The immature pods were surface sterilized with 70% ethanol for 1 min and followed by 0.1% $HgCl_2$ for 20 min and rinsed three times in distilled water. Immature seeds were removed from the pods and further experiments were carried out under sterile conditions. Immature seed was sterilized in by 70% ethanol for 1 min followed by 0.1% of $HgCl_2$ for 20 min and rinsed three times in sterile double distilled water. Immature zygotic embryo axis were excised and cultured on MS medium (Murashige and Skoog, 1962) with 18.1 µM/l 2,4-D., containing 3% sucrose and 0.85% agar for Induction of somatic embryogenesis. Somatic embryos derived from 60 day-old cultures of immature zygotic embryo axes raised in the presence of 18.1 µM/l 2,4-D were transferred to MS basal medium for germination.

Somatic embryos at various germinating stages corresponding to similar stages of germinating zygotic embryo axes were collected at different intervals of 5 - 40 days of culture on MS basal medium and used for protein extraction. In order to have representation of the profile of the entire germinating zygotic and somatic embryos, tissue slices cut horizontally along the length of germinating zygotic and somatic embryos were used for protein extraction in order to have a representation of the whole tissue of germinating embryos.

Protein extraction

All the samples mentioned above were weighed 100 mg each and ground in a pre-chilled motor and pestle in 1 ml of 50 mM Tris Hcl buffer (pH 5.7) containing 5 mM $MgCl_2$, 2mM K_2HPO_4, 1mM EDTA, 5 mM DTT, 2 mM KH_2PO_4, 5 mM DTT, 2% PVP, 20% glycerol, 1 mM NaF, 10 mM β-mercaptoethanol, 2 mM PMSF and while extracting 0.5 ml of n-hexane (per 100 mg sample) was added to the sample. After homogenization, the samples were centrifuged at 4°C for 20 min at 12,000 rpm. The supernatant was taken and soluble protein content was estimated by Lowry's (1951) method with minor modifications as given below

Protein estimation by Lowry's method

Solution A consisted of 4% sodium carbonate in 0.2 N sodium potassium tartrate, Solution B consisted of 1% cupric sulphate, and Solution C was 2% Sodiumpotassium tartrate and Solution D was 1N Folin's reagent (commercial). The working solution was obtained by mixing solutions A, B, C in a ratio of 23:1:1 and this solution were used within 24 h of preparation. One ml of the working solution was added to one ml of protein sample, mixed well and allowed to stand for 1 min. Then 0.2 ml of solution D was added rapidly while vortexing the sample. After 30 min, absorbance of the sample was recorded at 750 nm. Bovine serum albumen (BSA Fraction V) was used as a standard protein (5 - 50 µg).

SDS-PAGE

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970) with minor modifications. The separation of proteins was performed in 5% stacking gel and 10% resolving gel. Both the resolving and stacking gel contained 2.4% bisacrylamide as a cross linker and 0.1% SDS. The final buffer concentrations were 0.45 M Tris Hcl pH (8.9) in resolving gel and 0.2 M Tris HCl (pH 6.7) in stacking gel. Ammonium persulphate and N,N,N,N-tetra methylethylene diamines (TEMED) were used as polymerizing reagents in final concentration of 0.05 and 0.1% respectively. The electrode buffer comprised of 0.0247 M Tris HCl and 0.19 M glycine and 0.1% SDS in one liter of distilled water (pH 8.3). The samples were mixed with sample buffer consisting of 0.5 M Tris Hcl (pH 6.8) and boiled at 90°C for 3 min. The samples (10 - 20 µl) having 50 µg protein were loaded in slab gel wells of the gel of 8 x 8 x 0.1 cm dimension which was polymerized in plain glass plates and was fixed to Broviga (India) vertical slab gel apparatus. Gels were run at room temperature at a voltage of 75 and 100 DC (direct current) for stacking and resolving gel, respectively. Electrophoresis was carried out until the bromophenol blue dye marker reached about 3-4 mm from the bottom of the gel. Then the gels were removed, and stained overnight with 0.25% (w/v) Commassie Brilliant Blue R 250 in methanol: glacial acetic acid: water (50:7:43) v/v. Medium range molecular weight marker (Bangalore Genei, Banglore, India, Pvt. Ltd.) was used for calibration.

Figure 1a. Different stages of germinating zygotic embryo axes obtained after different intervals of 7 days after culture on MS basal medium.

Figure 1b. Different stages of germinating somatic embryo obtained after different intervals of 40 days after culture on MS basal medium.

Protein phosphorylation

Collection of the sample, protein extraction and protein estimation was similar as for the SDS-PAGE. Protein phosphorylation was carried out in 3 steps. Phosphorylation of the protein sample, separations of the protein samples by SDS-PAGE as described previously and exposing the gels to X-ray films.

Total protein of 20 µg (10-20 µl) was taken and mixed in 5x reaction mixture with 1 M Tris HCl containing 1 M $MgCl_2$, and 0.1 M DTT. The reaction was initiated by adding 4 µCi [α-^{32}P] ATP to the reaction mixture containing the sample and incubated at room temperature. The reaction was terminated after 60 s by addition of SDS PAGE sample buffer.

SDS-PAGE was performed as described previously and stained with commassie Brilliant Blue R 250 in 0.25% w/v methanol, glacial acetic acid and water in ratios of (50:7:43) and dried.

The dried gels were exposed to the Kodak X-ray films by keeping them in the cassettes with intensifying screens and stored at -20°C for a week, then the X-ray films were developed and fixed with commercially available developer and fixer. Autoradiograms obtained were compared with the dried gels for determination of accurate molecular weights of the proteins.

RESULTS

Protein profiles at various stages of germination of somatic embryos were compared with germinating zygotic embryos. Cotyledonary staged somatic embryos isolated from 60 day-old cultures were placed on MS basal medium for germination. Various stages of germinating stages of zygotic embryos were obtained within 7 days of culture on basal medium (Figure 1a). However, germinating stages of somatic embryos comparable to zygotic embryos in terms of morphology were obtained at different intervals of 40 days of culture on basal medium (Figure 1b). Many proteins which were expressed in cotyledonary somatic embryos (isolated from 30 day old cultures) were also present in zygotic embryos obtained from mature, dry seed (Figure 3).

However, the intensity of 22-kDa proteins was much higher in zygotic embryos in comparison to cotyledonary somatic embryos isolated from 60-day-old cultures. The intensity of 65 kDa protein decreased at 2nd stage and almost undetectable in the subsequent stages of germination of zygotic and somatic embryos. Protein of 22 kDa decreased in intensity at 3rd stage of germination of zygotic embryos and the intensity further decreased as the germination progressed. However, in germinating somatic embryos, this protein decreased in intensity in

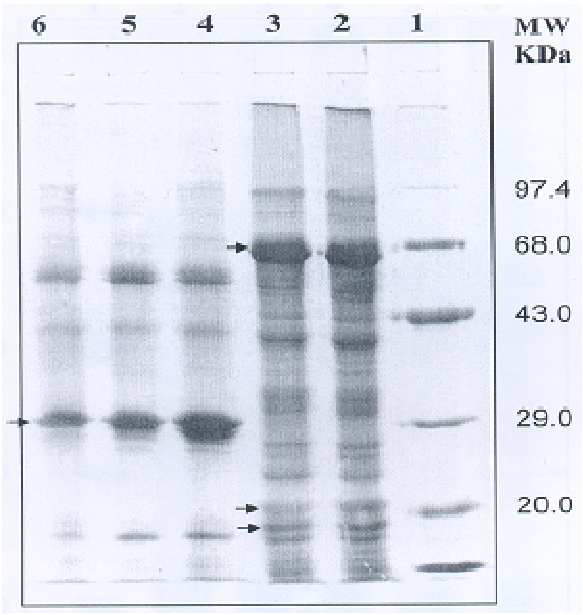

Figure 2. Protein patterns of somatic embryos during different stages of germination. Lane.1, Molecular weight marker in kilodaltons; Lane 2, somatic embryos derived from 60 day-old culture; Lanes3-6, different germinating stages of somatic embryos.

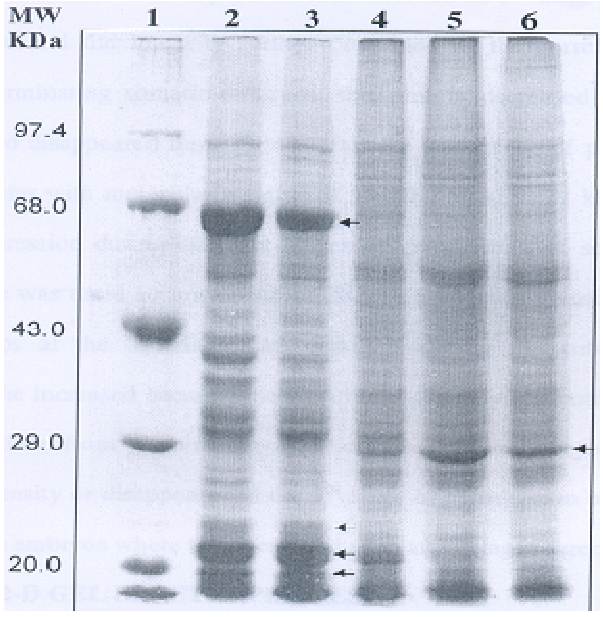

Figure 3. Protein patterns of embryo axes during different stages of germination. Lane 1, Molecular weight marker in kilodaltons; Lane 2, zygotic embryo axes derived from mature, dry seed; Lanes 3-6, different germinating stages of zygotic embryo axes.

the second stage and disappeared immediately after the emergence of plumule and radicle (Figure 2). Proteins of 55, 53, 27 and 25 kDa proteins did not vary in expression

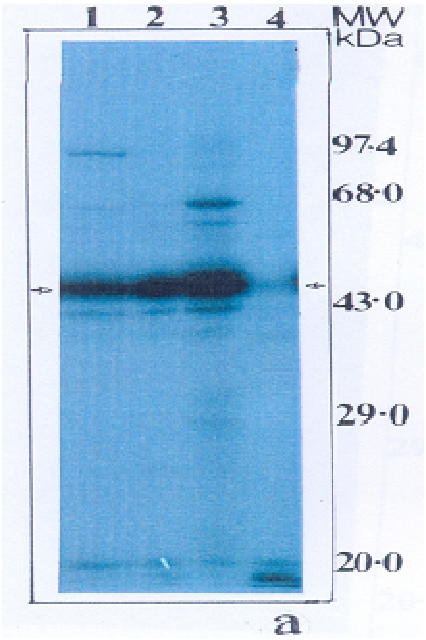

Figure 4a. Changes in protein phosphorylation pattern in different stages of germinating somatic embryos on MS basal medium. Total protein extracted from different germinating stages of somatic embryos were labeled by addition of 4 µCi[γ-^{32}P] ATP in phosphorylation reaction mixture at room temperature, separated by SDS-PAGE and exposed to X-ray films for 5 days. Lane 1, cotyledonary somatic embryos derived from 60 day old culture; Lanes 2-4, different stages of germinating somatic embryos.

during different stages of germination of somatic and zygotic embryos. There was rapid accumulation of 28 kDa protein in germinating zygotic and somatic embryos at the 3rd stage and persisted during the subsequent stages of germination. The increased accumulation could be possibly attributed to emergence of radicle and plumule from germinating zygotic and somatic embryos. Many proteins decreased in intensity or disappeared at the 3rd stage of germination of somatic embryos unlike in zygotic embryos where they persisted even at 5th stage of germination.

Same samples were taken to study changes in protein phosphorylation of various stages of germinating somatic and zygotic embryos. A clear difference was observed in pattern of protein phosphorylation in germinating somatic and zygotic embryos. A phosphoprotein of 50 kDa that was heavily phosphorylated in mature zygotic embryos at second stage of germination disappeared in 3rd stage of germination. In contrast, germinating somatic embryos showed an enhanced phosphorylation of 50 and 43 kDa proteins until 3rd stage followed by a drastic reduction in the level of phosphorylation at 4th stage (Figure 4a,b). In

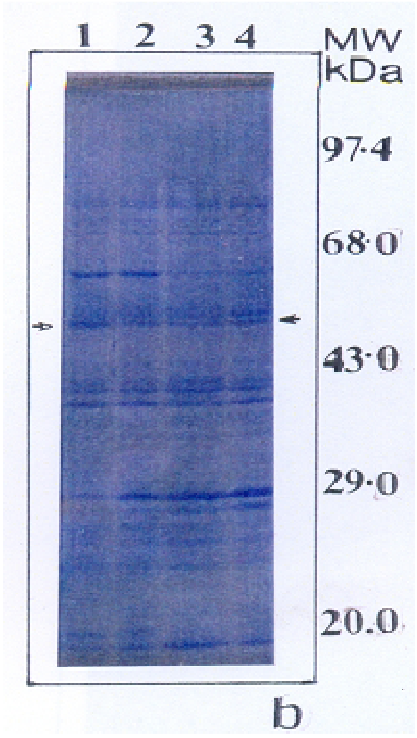

Figure 4b. SDS-PAGE analysis of total proteins of different stages of germinating somatic embryos. Lane 1, Cotyledonary somatic embryos isolated from 60 day old culture; Lanes 2-4, different stages of germinating somatic embryos.

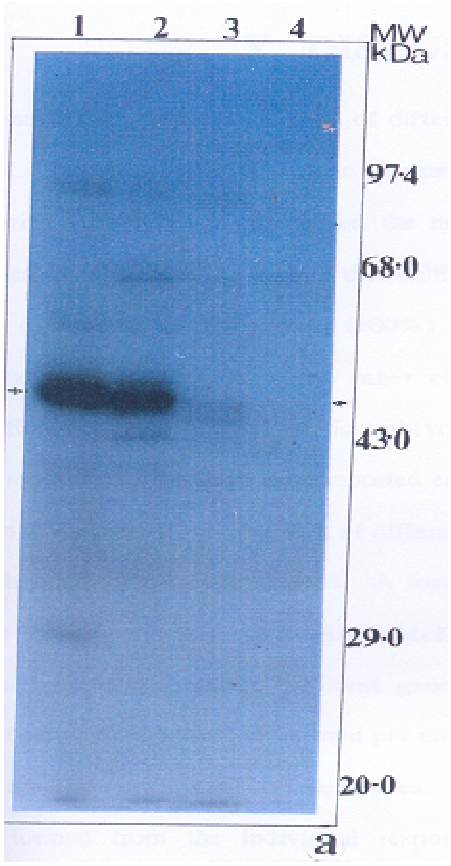

Figure 5a. Changes in protein phosphorylation pattern of germinating zygotic embryo axes are cultured on MS basal medium. Total protein extracted from different germinating stages of zygotic embryo axes were labeled by addition of 4 µCi[γ- ^{32}P] ATP in phosphorylation reaction mixture at room temperature, separated by SDS-PAGE and exposed to X-ray films for 5 days. Lane 1, Mature zygotic embryo axes; Lanes 2-4, different stages of germinating zygotic embryo axes.

addition, phosphoproteins of 68 and 65 kDa specifically appeared in the 3rd stage of germination of somatic embryos and were not observed during any germinating stages of zygotic embryos (Figure 5a, b).In addition, phosphoproteins of 97, 65, 61 and 43 kDa exhibited differential phosphorylation during different stages of germination of zygotic and somatic embryos.

DISCUSSION

Zygotic and somatic embryos displayed differences with regard to expression of many proteins that were detectable as clear bands during later stages of germination of zygotic embryos whereas they appeared in low intensity or absent in the corresponding stages of somatic embryos. A rapid decline in 65 kDa proteins observed immediately following germination of somatic and zygotic embryos indicates that it is a storage protein. Like zygotic embryos, the availability of food reserves, presence of germination associated proteins and phytohormones regulate germination and conversion of somatic embryos to seedlings. For examples, in alfalfa, increased levels of storage proteins and free amino acid accumulation, but not starch, were correlated with increased rate of somatic embryo germination and conversion and increased vigour of seedlings (Horbowioz et al., 1995; Lai and Mckersie, 1994a). Faure and Aarrouf (1994) compared the metabolism of starch and lipids in somatic and zygotic embryos of grapevine; one remarkable difference was that somatic embryos of somatic genotypes were unable to use their reserves and this possibly led to their inability to germinate into plantlets. Komatsuda (1992) reported that soybean seed storage proteins (β-conglycinin and glycinin) accumulated in somatic embryos under tissue specific and stage specific control analogous to that in zygotic embryos. These proteins quickly disappeared from somatic embryo cotyledons during germination in a fashion similar to that for zygotic embryos Xiang et al. (2007) also detected similar proteins during somatic embryogenesis in ***Freesia refracta*** differential pattern

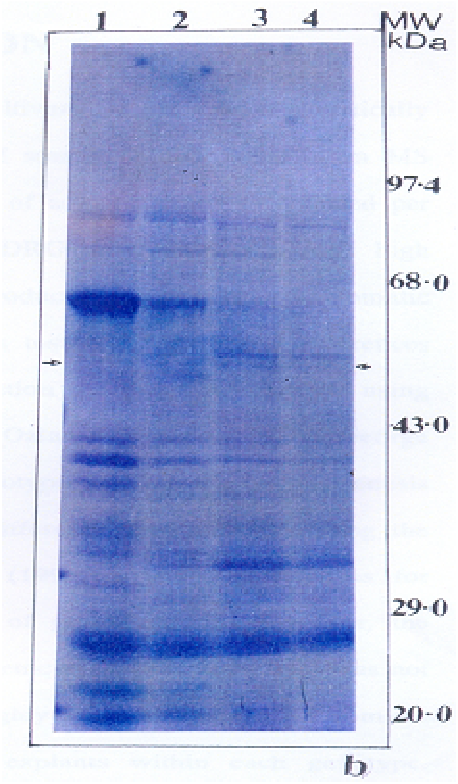

Figure 5b. SDS-PAGE analysis of total proteins of different stages of germinating zygotic embryo axes. Lane 1, Mature zygotic embryo axes; Lanes 2-4, different stages of germinating zygotic embryo axes.

observed for 50 kDa proteins during the first three germinating stages of zygotic and somatic embryos imply that bio-chemical events that follow germination are not completely identical for somatic and zygotic embryos.

However, a complete absence of 50 kDa phosphorprotein in 4th stage of germination of zygotic embryos while its presence at a minimum level in the corresponding stage of somatic embryos suggests that phosphorylation/dephosphorylation of this protein plays an important role in later stages of germinating somatic and zygotic embryos. The network of post-transcriptional and post-translational modifications ensures temporally and spatially appropriate patterns of downstream related gene expression (Elisabetta et al., 2008).

The analysis of protein synthesis in developing embryos from different plant species shows that is possible to distinguish group of polypeptides specific to the different developmental stage. One of these groups included polypeptides that became abundant during zygotic embryo maturation period of cotton (Galauga et al., 1981), wheat (Quatrano, 1986), maize (Sanchez Matinez et al., 1986) and rapidly disappear during early germination. This is an attempt made to investigate changes in protein phosphorylation during different developmental stages of somatic and zygotic embryogenesis in groundnut, which would help us in various morphological, physiological, and genetically related studies of embryogenesis in groundnut. This study also provides us the knowledge of storage proteins and LEA (late embryogenic) proteins, which help in overcoming the problems of seed dormancy and germination in various cotyledonary and dicotyledonary plant species.

REFERENCES

Baker CM, Wetzstein HY (1992). Somatic embryogenesis and plant regeneration from leaflets of peanut, ***Arachis hypogaea L***. Plant Cell Rep. 13: 159-163.

Baker CM, Burns A, Wetzstein HY (1994). Influence of photoperiod and medium formulation on peanut somatic embryogenesis. Plant Cell Rep. 13: 159-163

Baker CM, Durham RE, Burns RA, Parrott WA, Wetzstein HY (1995). High frequency somatic embryogenesis in peanut (***Arachis hypogaea L.***) using mature dry seed. Plant Cell Rep. 15: 38-42.

Baker CM, Wetzstein HY (1998). Leaflet development, induction time and medium influence somatic embryogenesis in peanut (***Arachis hypogaea L.***). Plant cell Rep. 17: 925-92

Chengalrayan K, Satheya SS, Hazra S (1994). Somatic embryo-genesis from mature embryo-derived leaflets of peanut (***Arachis hypogaea*** L). Plant Cell Rep. 13: 578-581.

Chengalrayan K, Mhaske VB, Hazra S (1997). High-frequency conversion of abnormal peanut somatic embryos. Plant Cell Rep. 16: 783-786.

Durham RE, Parrott WA (1992). Repetitive somatic embryogenesis from peanut cultures in liquid medium. Plant Cell Rep. 11: 122-125.

FAO (2007). Quarterly Bulletin of Statistics, vol. 8(3/4), FAO, Rome.

Johanson EL, Smith BW (1956). ***Arachis hypogaea*** x ***Arachis diogoi*** embryo and seed failure. Am. J. Bot. 43: 250-258.

Kocher G (1996). Molecular markers and genome mapping. In: Current Status of Agricultural Biotechnology in Indonesia (Darussamin A., ed.), AARD, Jakarta, pp. 89-108.

Eapen S, George L (1993). Somatic embryogenesis in peanut: Influence of growth regulators and sugars. Plant Cell Tiss. Org. Cult. 35: 151-156.

Elisabetta M, Anna M, Cristina C, Davide G, A Michele S, Luigi C (2008). Abiotic stress response in plants: When post-transcriptional and post-translational regulations control transcription Plant. Science, Vol. 174, Issue 4, April 2008, pp. 420-431.

Faure O, Aarrouf J (1994). Metabolism of reserve products during development of somatic embryos and germination of zygotic embryos in grapevine. Plant Sci. 96: 167-178.

Galauga GA, Legocki AB, Greenway SC, Dure LS (1981). Cotton messenger RNA sequences exist in both polyadenylated and nonpolyadenylated forms. J. Biol. Chem. 256: 2551-2560.

Gill R, Saxena PK (1992). Direct somatic embryogenesis and regeneration of plants from seedling explants of peanut (***Arachis hypogaea*** L.): Promotive role of thidiazuron. Can. J. Bot. 70: 1186-1192.

Horbowioz M, Obendorf RL, McKersie BD, Viands DR (1995). Soluble saccharides and cyclitols in alfalfa (***Medicago sativa*** L.) somatic embryos, leaflets and mature seeds. Plant Sci. 109: 191-198.

Komatsuda T (1992). Research on somatic embryogenesis and plant regeneration in soybean. Bull. Natl. Inst. Agriobiol. Resour. 7: p. 1.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 277: 680-685.

Lai F, McKersie BD (1994a). Regulation of starch and protein accumulation in alfalfa (***Medicago sativa*** L.) somatic embryos. Plant Sci. 100: 211-219.

Little EL, Magbanua ZV, Parrott WA (2000). A protocol for repetitive somatic embryogenesis from mature peanut epicotyls. Plant Cell Rep. 19: 351-357.

Lowry OH, Rosebrouch NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol regeant. J. Biol. Chem. 108: 1203-1210.

Mckently AH (1991). Direct somatic embryogenesis from axes of mature peanut embryos. In vitro: Cell Dev. Biol. 27P: 197-200.
Mroginski LA, Kartha K, Shyluk JP (1981). Regeneration of peanut (***Arachis hypogeae L.***) plantlets by invitro culture of immature leaves. Can. J. Bot. 59: 826-830.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
Ozais-Akins P (1989). Plant regeneration from immature embryos of peanut. Plant Cell Rep. 8: 217-218.
Quatrano RS (1986). Regulation of gene expression by abscisic acid during angiosperm embryo development. Oxford Surv. Plant Mol. Cell Biol. 3: 467-477.
Reddy LR, Reddy GM (1993). Factors affecting direct somatic embryogenesis and plant regeneration in groundnut, ***Arachis hpogaea L***. Ind .J. Exp. Biol. 31: 57-60.
Rey HY, Scocchi AM, Gonzalez AM, Mroginski LA (2000). Plant regeneration in ***Arachis pintoi*** (Leguminosae) through leaf culture. Plant Cell Reports, 19: 856-862.
Roja RA, Padmaja G (2005). A Protocol for high frequency plant conversion rom somatic embryos of peanut(***Arachis hypogaea.L***)cv DRG-12) J. Plant Biotech. 7(3): 187-193.
Sanchez-Martinez D, Puigdomenech P, Pages M (1986). Regulation of gene expression in developing ***Zea mays*** embryos. Plant Physiol. 82: 543-549.
Venkatachalam P (1996). Tissue culture, mutational and genetic studies in groundnut (***Arachis hypogaea*** L.) to develop cultivar for high yield, oil content and disease resistance. Ph.D. Thesis, Bharathidasan University, Indian.
Venkatachalam P, Kavi Kishor PB, Jayabalan N (1997). High frequency somatic embryogenesis and efficient plant regeneration from hypocotyls explants of groundnut (***Arachis hypogaea*** L.). Curr. Sci. 72: 271-275.
Wetzstesin HY, Baker CM (1993). The relationship between somatic embryo morphology and conversion in peanut (***Arachis hypogaea*** L.). Plant Sci. 92: 81-89.
Xiang GAO, Yue-jun HUI, Yi-qun XUE, Yang FU, Man AO, Li WANG (2007). Specific Proteins in the Indirect Somatic Embryogenesis of ***Freesia Refracta*** Chemical Research in Chinese Universities Vol. 23, Issue 2, March 2007, pp. 187-190.