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Changes in protein profiles associated with somatic embryogenesis in peanut

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Abstract

The somatic embryogenesis potential of zygotic embryo axes of peanut (*Arachis hypogaea* L. cv. DRG-12) at different stages of development was evaluated by culturing on MS medium with 18.1 μM 2,4-dichlorophenoxyacetic acid (2,4-D). A 100 % frequency with 18.3 somatic embryos per explant was observed from 4 mm long immature zygotic embryo axes collected 31 - 40 d after pollination. Medium supplemented with 16.6 μM picloram resulted in slow development of somatic embryos whereas in the presence of 21.5 μM α-naphthaleneacetic acid (NAA), the explants underwent maturation with induction of roots after 30 d. The changes in protein profiles in zygotic embryo axes at different stages of development correlated with their potential to form somatic embryos. Immature zygotic embryo axes exhibited high frequency somatic embryogenesis in the stage preceding abundant accumulation of 22 and 65 kDa proteins. The content of 22 and 65 kDa proteins decreased immediately after culture on medium fortified with 18.1 μM 2,4-D and increased again after 12 d of culture coinciding with the development of somatic embryos on the explants. The content of 22 and 65 kDa proteins was low at 15 d of culture on medium supplemented with 16.6 μM picloram possibly due to slow development of the somatic embryos on the explant. On maturation medium containing 21.5 μM NAA, a marked increase in the content of 22 and 65 kDa proteins in 15 d-old cultures was observed.

Additional key words: protein analysis, sodium dodecyl sulphate-polyacrylamide gel electrophoresis, zygotic embryo axes.

Introduction

Peanut or groundnut (*Arachis hypogaea* L.) is one of the principal oil seed legumes rich in protein and is grown in tropical and sub-tropical regions of the world. Plant regeneration *via* organogenesis (Mroginski *et al*. 1981, Chengalrayan *et al*. 1995, Victor *et al*. 1999) and somatic embryogenesis (Sellars *et al*. 1990, Baker and Wetzstein 1994, Chengalrayan *et al*. 1998, Little *et al*. 2000, Radhakrishnan *et al*. 2002, Joshi *et al*. 2003) has been reported from different explants of peanut. Regeneration of plants *via* somatic embryogenesis is of special significance as it yields genetically more stable regenerants owing to single cell origin. Somatic embryogenesis also holds considerable potential for enhancing the understanding of various aspects related to embryo development and provides the most efficient regeneration method suitable for gene transfer. In peanut, immature zygotic embryos, cotyledons and developing leaves have been reported to be the most responsive explants for the induction of somatic embryogenesis compared to the differentiated plant tissues (Hazra *et al*. 1989, George and Eapen 1993, Reddy and Reddy 1993). Many other factors including genotype, photoperiod, nitrogen formulation, type and concentration of auxin, carbon source supplied in the medium are also known to affect the embryogenesis response from somatic tissues of peanut (Chengalrayan *et al*. 1998, Ozias-Akins *et al*.

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; MS - Murashige and Skoog (1962) medium; Mr - molecular mass; NAA - α-naphthaleneacetic acid; PAGE - polyacrylamide gel electrophoresis; SDS - sodium dodecyl sulphate; Tris - Tris hydroxymethyl aminomethane.
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1992, Eapen and George 1993, Baker *et al*. 1994, McKently 1995). Although the morphological and histological development of *in vitro* somatic embryogenesis have been characterized in peanut, biochemical aspects of somatic embryogenesis have so far not been elucidated.

There are relatively few studies that have examined the biochemical characteristics of explants in relation to their competence for somatic embryogenesis. Roberts *et al*. (1989) used a combination of SDS-PAGE and microscopy to characterize stages of white spruce embryo development in order to develop more precise criteria for somatic embryogenesis. Their study revealed that the competence to form somatic embryos is limited to specific stage of development prior to the accumulation of storage proteins. Buckley and Trigiano (1994) compared the embryogenic potential of *Cercis canadensis* (redbud) ovules cultured during different developmental stages with protein profiles of ovules over time. Differences in staining intensity of six bands have been found to be associated with changes in the somatic embryogenic potential of ovules. The objective of the present study was to correlate the changes in protein profiles in zygotic embryo axes of different developmental stages with their somatic embryogenesis potential. An attempt has also been made to study the changes in protein patterns during induction of somatic embryogenesis and maturation in cultured immature zygotic embryo axes.

Materials and methods

Seeds of peanut (*Arachis hypogaea* L. cv. DRG-12) were collected from Directorate of Oilseeds Research, Hyderabad. The cultivar DRG-12 is a high yielding Spanish bunch type and matures in 110 - 115 d in post-rainy season. Immature pods were harvested from field-grown plants at different days after pollination and washed under running tap water and rinsed in distilled water. The pods were sterilized with 70 % ethanol for 1 min followed by 0.1 % mercuric chloride for 20 min and rinsed three times in distilled water. The isolated immature seeds were sterilized with 70 % ethanol for 1 min followed by 0.1 % $HgCl_2$ for 15 min and rinsed thoroughly in distilled water under aseptic conditions. Somatic embryogenesis was induced from immature zygotic embryo axes as reported by Reddy and Reddy (1993). The embryo axes were aseptically removed from immature seeds and sorted into 5 groups according to the size (2 - 6 mm) corresponding to different days after pollination. They were placed in culture tubes (25 × 150 mm) containing 15 cm^3 of medium, and two explants were cultured per culture tube. The somatic embryogenesis from zygotic embryo axes of different sizes was evaluated by placing on MS (Murashige and Skoog 1962) medium with 3 % sucrose, 18.1 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.85 % agar type 1 (*Himedia*, Mumbai, India). The culture medium was supplemented individually with 18.1 µM 2,4-D, 16.6 µM picloram (4-amino-3,5,6-trichloropicolinic acid) and 21.5 µM α-naphthaleneacetic acid (NAA) for observing their effects on induction of somatic embryogenesis from 4 mm long immature zygotic embryo axes. The cultures were incubated at 25 ± 2 °C under a 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 57 µmol m^{-2} s^{-1} provided by white fluorescent tubes. Twenty explants were used for each treatment with three replicates. The frequencies of explants in which somatic embryos were formed and the number of somatic embryos induced per explant were determined after four weeks of culture.

Mature seed (obtained from mature dry pod) was subjected to sterilization as mentioned above and embryo axes were isolated after soaking them in sterilized distilled water for half an hour. Total protein was extracted from mature zygotic embryo axes along with immature zygotic embryo axes of different sizes (as described above) and subjected to electrophoresis. The samples of immature zygotic embryo axes (4 mm long) cultured on MS medium supplemented individually with 18.1 µM 2,4-D and 16.6 µM picloram were collected at explant stage (0 h) and at different intervals of 6, 12, 24, 48 h followed by 3, 6, 9, 12 and 15 d of culture for studying the protein profiles associated with direct somatic embryogenesis. Protein was extracted from immature zygotic embryo axes (4 mm long) at explant stage (0 h) and after 6, 12, 24, 48 h and 3, 6, 9, 12, 15 d of culture on MS medium with 21.5 µM NAA for analyzing the protein profiles during maturation.

Protein extraction: All the samples mentioned above (100 mg each) were ground in a pre-chilled mortar and pestle in 1 cm^3 of 50 mM Tris HCl buffer (pH 7.5) containing 5 mM magnesium chloride, 2 mM dipotassium phosphate, 1 mM ethylenediaminetetraacetic acid, 5 mM dithiothreitol, 2 % polyvinyl pyrrolidone, 20 % glycerol, 10 mM sodium fluoride, 10 mM β-mercaptoethanol, 2 mM phenyl methyl sulphonyl fluoride and 0.5 cm^3 of *n*-hexane was added at the time of extraction. After homogenization, the samples were centrifuged at 4 °C for 20 min at 16 300 *g* and the supernatant was taken for calculating the protein content. The protein content was determined using Lowry (1951) method. Three samples of each stage were independently used for protein extraction.

Protein electrophoresis: SDS-PAGE was performed according to the method of Laemmli (1970). The protein samples were mixed with sample buffer consisting of 0.5 M Tris HCl (pH 6.8), 10 % glycerol, 4 % mercapto-ethanol, 2 % SDS and bromophenol blue as a tracking dye. The preparations were boiled at 90 °C for 3 min and

then stored at -80 °C. The samples (0.01 - 0.02 cm^3) having 50 µg proteins were loaded in the wells of 0.8 mm thick SDS-PAGE gel. The stacking gel consisted of 5 % polyacrylamide with 0.5 M Tris HCl (pH 6.8) and resolving gel had 10 % polyacrylamide with 3.0 M Tris-HCl (pH 8.9) each containing 0.1 % SDS. The electrode buffer consisted of 0.025 M Tris-HCl (pH 8.3), 0.19 M glycine and 1 % SDS. The samples were run at constant current of 70 - 75 V for 1 h and then the voltage was increased to 110 V for 5 - 6 h in resolving gel. The gels were stained overnight with 0.25 % (m/v) Commassie Brilliant Blue R 250 in methanol, glacial acetic acid and water in a ratio of 50:7:43 (v/v), and subsequently destained with solution containing 5 % methanol and 7.5 % acetic acid. Medium range molecular mass marker (*Genei Pvt. Ltd.*, Bangalore, India) was used for calibration. Protein analyses were repeated thrice using samples from different treatments; at least 3 gels were performed per sample per treatment.

Results

Direct somatic embryogenesis was induced from 2 - 6 mm long immature zygotic embryo axes cultured on MS medium supplemented with 18.1 µM 2,4-D. The frequency, duration and the number of somatic embryos induced per explant were greatly influenced by the size of zygotic embryo axes (Table 1). Immature zygotic embryo axes of 2 mm long responded with a frequency of 74.2 % and somatic embryo induction occurred after 20.9 d of culture and those smaller than 2 mm turned brown after culture. The maximum frequency of somatic embryogenesis (100 %) was observed from 4 mm long immature zygotic embryo axes with 18.3 somatic embryos per explant (Fig. 1*A*). Induction of somatic embryogenesis was found to be asynchronous with the first detectable somatic embryos appearing after 10.2 d of culture and their number substantially increased (18.3 per explant) after 30 d of culture. The frequency of somatic embryogenesis decreased (53.3 %) in 6 mm long immature zygotic embryo axes with 6.5 somatic embryos per explant at the end of 30 d of culture. Somatic embryos were induced from 4 mm long immature zygotic embryo axes after 10.2 d of culture whereas a delayed response (18.4 - 20.9 d) was observed in 2 - 3 mm long zygotic embryo axes. Somatic embryos were not produced in mature zygotic embryo axes on 18.1 µM 2,4-D; instead, callus proliferated from the base in 80 % of the explants.

Table 1. Induction of somatic embryogenesis from zygotic embryo axes of different developmental stages on MS medium with 18.1 µM 2,4-D. Means ± SE of three replicates, with each replicate consisting of 20 explants. Means followed by the same letter in a column are not significantly different ($P < 0.05$) according to Newman-Keul's multiple range test.

Size [mm]	Time after pollination [d]	Somatic embryogenesis [%]	Number of somatic embryos [explant^{-1}]	Average duration for induction [d]
2	20 - 25	74.2 ± 2.01^{c}	5.5 ± 0.38^{e}	20.9 ± 0.49^{a}
3	26 - 30	86.7 ± 1.05^{b}	13.3 ± 0.16^{b}	18.4 ± 0.35^{b}
4	31 - 40	100.0 ± 0.00^{a}	18.3 ± 0.12^{a}	10.2 ± 0.19^{d}
5	41 - 50	69.2 ± 2.01^{d}	9.9 ± 0.19^{c}	11.3 ± 0.55^{d}
6	51 - 60	53.3 ± 1.67^{e}	6.5 ± 0.15^{d}	14.2 ± 0.27^{c}

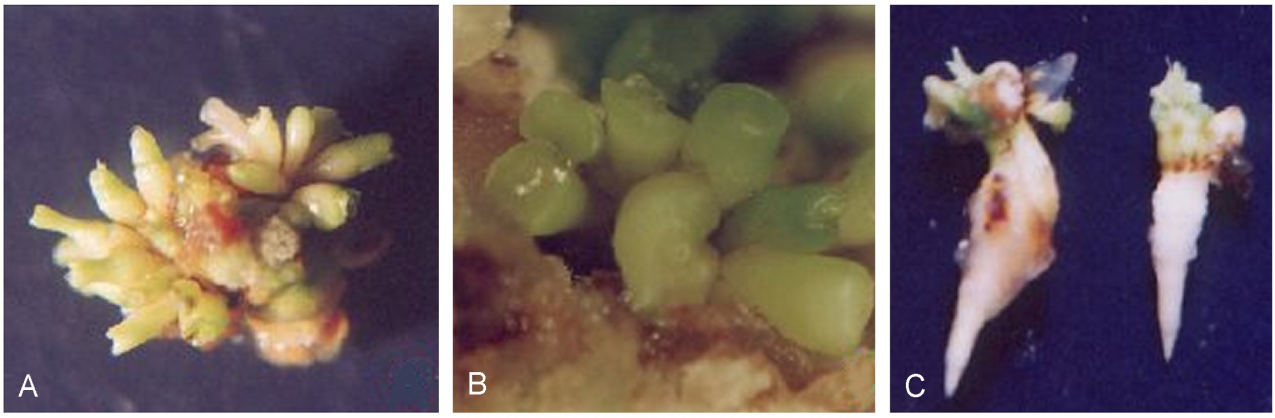

Fig. 1. *A* - Somatic embryos formed on immature zygotic embryo axes cultured on MS medium with 18.1 µM 2,4-D after 30 d of culture. *B* - Somatic embryos formed on immature zygotic embryo axes cultured on MS medium with 16.6 µM picloram after 30 d of culture. *C* - Induction of roots from immature zygotic embryo axes after 30 d of culture on MS medium with 21.5 µM NAA.

The response of somatic embryogenesis from 4 mm long immature zygotic embryo axes cultured on medium supplemented individually with 16.6 µM picloram and 21.5 µM NAA was evaluated. Somatic embryogenesis was induced with a frequency of 100 % in the presence of 16.6 µM picloram but the average number of somatic embryos were less (10.4 per explant) than on 2,4-D medium. The development of somatic embryos was slower and torpedo staged somatic embryos were predominantly observed in 30 d-old cultures on picloram (Fig. 1*B*) unlike on 2,4-D medium where at the same time somatic embryos of different morphological classes were observed. There was no induction of somatic embryogenesis from immature zygotic embryo axes cultured on 21.5 µM NAA. The explants underwent maturation during the first two-weeks of culture and subsequently epicotyl and hypocotyl regions expanded with induction of multiple leaflet like structures and root at the end of 30 d (Fig. 1*C*).

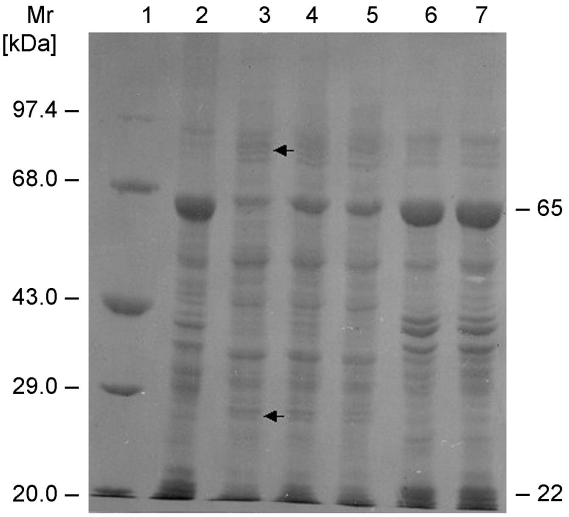

Fig. 2. SDS-PAGE analysis of total proteins of zygotic embryo axes of different developmental stages. Lane 1 - molecular mass marker (Mr), 2 - mature zygotic embryo axes, 3-7 - immature zygotic embryo axes of various sizes (2, 3, 4, 5 and 6 mm, respectively). The bands of interest are pointed *on the right*.

SDS-PAGE analysis was made on proteins extracted from immature zygotic embryo axes of different sizes along with mature zygotic embryo axes for correlating the changes in protein profiles with the competence to form somatic embryos (Fig. 2). The most noticeable change was observed with respect to proteins of 22 and 65 kDa, which were expressed in relatively low amounts in 2 - 4 mm long immature zygotic embryo axes and increased in abundance during later stages of development. Similarly, proteins of 40, 38 and 35 kDa were detectable as faint bands in 2 - 4 mm long immature zygotic embryo axes and the content of these proteins significantly increased in 5 - 6 mm long immature zygotic embryo axes. Subsequently, the content of these proteins decreased in mature zygotic embryo axes. On the contrary, proteins of 81 and 26 kDa appeared in 2 - 4 mm long zygotic embryo axes and disappeared in zygotic embryo axes excised at later stages of development. The increased accumulation of 22 and 65 kDa proteins during later stages of zygotic embryogenesis was correlated with the decrease or loss of their potential to form somatic embryos on MS medium with 18.1 µM 2,4-D.

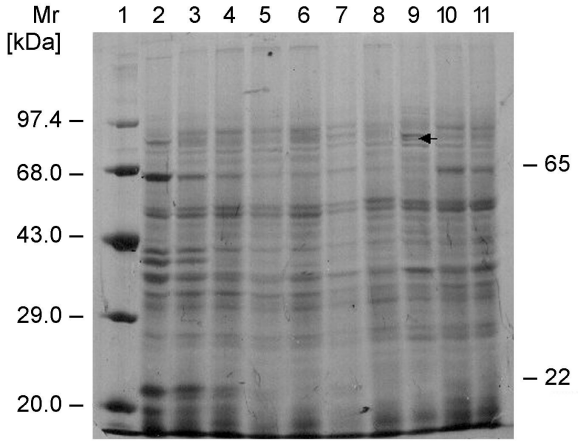

Fig. 3. SDS-PAGE analysis of total proteins of immature zygotic embryo axes (4 mm long) at explant stage (0 h) and after culture on MS medium with 18.1 µM 2,4-D for different durations. Lane 1 - Mr, 2 - immature zygotic embryo axes, 3-11 - immature zygotic embryo axes cultured for 6, 12, 24, 48 h followed by 3, 6, 9, 12 and 15 d, respectively. The bands of interest are pointed *on the right*.

Changes in protein patterns during induction of somatic embryogenesis were studied by harvesting the immature zygotic embryo axes at different durations of culture on medium containing 18.1 µM 2,4-D. A 81 kDa protein was found only in 9 d-old cultures and disappeared during later stages of culture (Fig. 3). Proteins of 22 and 65 kDa decreased in content immediately after culture and increased again after 12 d of culture coinciding with the development of somatic embryos on the explant.

Protein patterns were studied in immature zygotic embryo axes cultured on MS medium supplemented with 16.6 µM picloram (Fig. 4). Somatic embryogenesis was induced within 11.3 d of culture and the development of somatic embryos progressed slowly in the presence of picloram. The content of 22 and 65 kDa proteins decreased immediately after 6 h of culture and expressed at very low quantities throughout the culture period. This is in contrast to 2,4-D supplemented medium where the content of these proteins increased again after 12 d of culture. Protein of 81 kDa appeared after 9 d of culture and persisted even in 15-d-old cultures. This could be possibly due to the slow development of somatic embryos observed on medium with picloram. Similarly, this protein was expressed in 2-4 mm long zygotic embryo axes and disappeared during later stages of zygotic

embryogenesis.

Immature zygotic embryo axes undergoing maturation on NAA exhibited considerable differences in the expression of 22 and 65 kDa proteins (Fig. 5). The relative amounts of 22 and 65 kDa proteins decreased immediately after 6 h of culture and increased again after 3 d of culture on NAA supplemented medium. The content of 22 and 65 kDa proteins significantly increased in 12 and 15 d-old cultures and could be associated with the maturation of the zygotic embryo axes. Proteins of 76, 50, 47 and 30 kDa were expressed more or less in the same amounts throughout the culture period in all samples of immature zygotic embryo axes cultured on medium containing either 2,4-D, NAA or picloram.

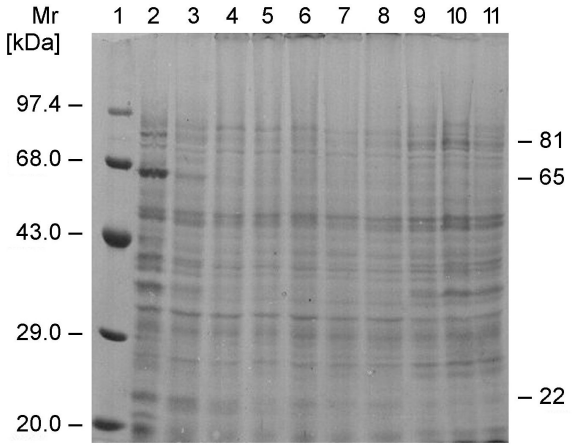

Fig. 4. SDS-PAGE analysis of total proteins of immature zygotic embryo axes (4 mm long) at explant stage (0 h) and after culture on MS medium with 16.6 µM picloram for different durations. Lane 1 - Mr, 2 - immature zygotic embryo axes, 3-11 - immature zygotic embryo axes cultured for 6, 12, 24, 48 h followed by 3, 6, 9, 12 and 15 d, respectively. The bands of interest are pointed *on the right.*

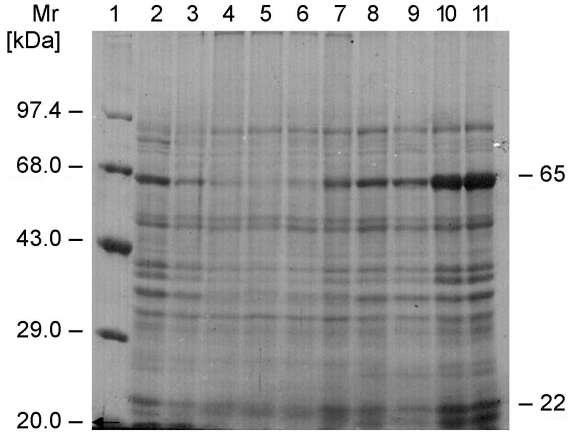

Fig. 5. SDS-PAGE analysis of total proteins of immature zygotic embryo axes (4 mm long) at explant stage (0 h) and after culture on MS medium with 21.5 µM NAA for different durations. Lane 1 - Mr, 2 - immature zygotic embryo axes, 3-11 - immature zygotic embryo axes cultured for 6, 12, 24, 48 h followed by 3, 6, 9, 12 and 15 d, respectively. The bands of interest are pointed *on the right*.

Discussion

In peanut, somatic embryogenesis can be induced relatively easily in young meristematic tissues such as immature embryos, cotyledons and developing leaves (Hazra *et al.* 1989, George and Eapen 1993, Reddy and Reddy 1993). In the present study, high frequency somatic embryogenesis (100 %) was observed from 4 mm long immature zygotic embryo axes with 18.3 somatic embryos per explant. Mature zygotic embryo axes cultured on MS medium with 18.1 µM 2,4-D failed to respond for somatic embryogenesis indicating that developmental stage of the explant plays an important role on induction of somatic embryogenesis in peanut. Similarly, Hazra *et al.* (1989) found that the size of immature peanut embryo axes (3 - 6 mm long) was critical in obtaining direct somatic embryogenesis. Baker *et al.* (1994) reported that stage III cotyledons (with visible embryo axes) of peanut were more preferable because of more uniform embryogenic response and also because of greater supply of explant material. Baker *et al.* (1995) and Chengalrayan *et al.* (1998) have reported that extensive cell proliferation was found to be necessary for regaining embryogenesis potential in mature zygotic embryo axes of peanut as the propensity of embryos to regenerate diminished with age. It is hypothesized that the expression of an embryogenic developmental pathway, a feature characteristic of early embryogenic cells, remains active in the cells of immature embryos until certain stages of development are completed, but their embryogenic development may be eventually replaced by other developmental programmes such as embryo maturation (Vergara *et al.* 1990).

In the present study, somatic embryogenesis was induced from immature embryo axes (4 mm long) only in the presence of 2,4-D and picloram whereas those cultured on NAA supplemented medium underwent maturation with induction of roots at the end of 30 d of culture. These observations suggest that some important physiological changes that are necessary for the induction of somatic embryogenesis take place in the immature zygotic embryo axes in the presence of 2,4-D and picloram while they do not occur in the presence of NAA even under the same culture conditions. From the numerous reports on somatic embryogenesis in peanut, it is evident that exogenously applied auxin, particularly 2,4-D played an important role in somatic embryo induction and development (Lakshmanan and Taji 2000). The exact mechanism underlying the auxin-induced somatic embryo formation is not understood. However,

some studies implicate certain auxin-induced cellular processes such as embryo-specific DNA methylation (Vergara *et al*. 1990), disruption of tissue integrity by interrupting cell-cell interaction (Smith and Krikorian 1989) and establishment of cell polarity (Parrott *et al*. 1991) in the induction of somatic embryogenesis. Involvement of specific auxin binding proteins in auxin-dependent somatic embryogenesis has been identified in pea explants (Jacobsen 1991).

The analysis of protein patterns carried out in zygotic embryo axes of different developmental stages revealed that the explants were more competent to form somatic embryos in the stage preceding abundant accumulation of 22 and 65 kDa proteins. Increase in relative abundance of 22 and 65 kDa proteins during later stages of maturation of zygotic embryo axes suggested that they are possibly storage proteins. This is further evidenced by the observation that there was a rapid decline in the levels of these proteins during germination of zygotic embryo axes (Roja Rani and Padmaja, unpublished results). In soybean, elevated levels of various storage proteins in cotyledonary stage zygotic embryos were associated with a decrease in somatic embryogenesis (Dahmer *et al*. 1992). Similarly, the embryogenesis potential of immature zygotic embryos of interior spruce was highest before significant amounts of storage proteins had accumulated (Roberts *et al*. 1989). The present study corroborates the findings of aforementioned reports that abundant accumulation of storage proteins delineates a stage of physiological and/or biochemical maturation of zygotic embryos in which their competency to form somatic embryos declines or is lost when cultured on MS medium with 18.1 µM 2,4-D.

The biochemical and molecular changes associated with somatic embryogenesis have been studied in several species such as rapeseed (Crouch 1982), carrot (Choi and Sung 1984, Dodeman and Ducreux 1996), rice (Chen and Luthe 1987), pea (Stirn and Jacobsen 1987), cotton (Shoemaker *et al*. 1987), *Trifolium* (McGee *et al*. 1989), *Dactylis glomerata* (Hahne *et al* 1988), coffee (Yuffa *et al*. 1994), *Camellia japonica* (Pedroso 1995), soybean (Stejskal and Griga 1995), barley (Stirn *et al*. 1995), sugarcane (Blanco *et al*. 1997) and birch (Hvoslef-Eide and Corke 1997). However, to our knowledge, no attempt has been made to investigate the underlying biochemical changes during the process of somatic embryogenesis in peanut. Our studies on protein patterns during induction of somatic embryogenesis in immature zygotic embryo axes on medium with 18.1 µM 2,4-D revealed a decrease in the content of 22 and 65 kDa proteins immediately after 6 h of culture. The content of these proteins increased again after 12 d of culture coinciding with the development of somatic embryos on the explant. In contrast, in immature zygotic embryo axes cultured on medium with 16.6 µM picloram, these proteins decreased in content immediately after culture and no detectable increase was noticed up to 15 d of culture possibly due to the slow development of somatic embryos in the cultures. From these results, it is hypothesized that 22 and 65 kDa proteins are rapidly metabolized immediately after culture on 2,4-D and picloram supplemented medium, and this metabolization induced by 2,4-D and picloram brings about a physiological change in the constituent cells of the explants conducive for expression of somatic embryogenesis. Stuart *et al*. (1988) reported that alfalfa somatic embryos induced on 50 µM 2,4-D exhibited little storage protein accumulation, while those induced on 10 µM 2,4-D exhibited enhanced levels of storage proteins, suggesting that the high auxin levels during induction led to carryover effect that suppressed storage protein gene expression and deposition.

A different pattern in the expression of 22 and 65 kDa proteins was observed in immature zygotic embryo axes cultured on NAA supplemented medium. The contents of 22 and 65 kDa proteins decreased immediately after 6 h of culture followed by an increase in 3 d-old cultures. A marked increase in the content of these proteins was observed in 15-d-old cultures coinciding with the maturation of the immature zygotic embryo axes. From these results it is interpreted that although 22 and 65 kDa proteins are rapidly metabolized immediately after culture on medium supplemented with NAA, it does not provide the right signal for the expression of somatic embryogenesis and rather promotes maturation of the explants leading to an enormous increase in the amounts of 22 and 65 kDa proteins in 15-d-old cultures. This is further substantiated by our earlier observation that proteins of 22 and 65 kDa increased in abundance during later stages of zygotic embryogenesis and this was associated with the decline or loss of potential to undergo somatic embryogenesis on medium supplemented with 18.1 µM 2,4-D. Dahmer *et al*. (1992) reported that somatic embryos of soybean induced on NAA-containing medium accumulated detectable levels of all maturation-specific marker proteins except the 7S β and 29 kDa soybean agglutinin antigen and were similar in most respects to the cultured zygotic embryos.

In conclusion, immature zygotic embryo axes of peanut cv. DRG-12 retained the potential to undergo somatic embryogenesis at high frequency in the stage preceding abundant accumulation of 22 and 65 kDa proteins. These proteins offer best candidates for assessing the development and maturation of somatic embryos.

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