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Changes in biochemical constituents, enzyme activities and protein profiles during root-shoot differentiation in callus culture of *Dioscorea alata*

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ABSTRACT

The changes in biochemical constituents and polypeptide (PP) bands were studied during root-shoot differentiation in *Dioscorea alata* (greater yam). Among different explants used - leaf, node, internode and tuber, node explants were found to be the best for induction and growth of callus. Depending on weights of fresh and dry callus, high growth and best callusing were observed on MS medium supplemented with 2.5 µM NAA. This high growth value callus was subsequently supplemented with various concentrations and combinations of growth regulators to identify the suitable regeneration media. Regeneration of roots occurred in 12-16 days and was the best on MS medium having 2.0 µM NAA and 0.5 µM IBA. Shoots regenerated in 16 days in MS medium supplemented with 2.0 µM BAP and 0.5 µM NAA. The biochemical constituents such as total soluble sugars, reducing sugars, total soluble proteins and total phenols decreased whereas free amino acids increased during root and shoot differentiation of *D. alata*. Activities of enzymes, viz. α-amylase, acid invertase, acid phosphatase, acid protease and peroxidase decreased during callus differentiation. While four PP bands (25.56, 24.35, 19.13 and 18.2 kDa) appeared during root differentiation, only three PP bands (53.7, 25.12 and 19.13 kDa) were noted during shoot differentiation. One common PP band (19.13 kDa) appeared during both root and shoot differentiation. There was disappearance of four common bands (89.13, 69.8, 36.3 and 27.43 kDa) during differentiation of root and shoot. To conclude, changes in biochemical constituents and expression of root/shoot specific PP may be used as markers to characterize differentiation pathway and to augment the selection of regenerating potential callus for rapid *in vitro* propagation.

Key words: *Dioscorea alata*, Metabolites, Polypeptide bands, Root differentiation, Shoot differentiation

Differentiation refers to the growth and morpho-physiological specialization of cells from unorganized mass of callus. It is a prerequisite for the application of tissue culture protocols for the crop improvement. Knowledge on the control of cell differentiation has hardly grown since the demonstration that differentiation of organized structures in tissue culture is influenced by growth regulators such as cytokinins and auxins along with other components of the culture medium (Smith and Krikorian 1991). Visible manifestations of cell differentiation include greening of callus, variation in cell wall thickness and appearance of certain cytoplasmic organelles such as plastids. As organized growth of callus cultures leading to root and shoot differentiation is preceded by changes at the cellular and molecular level, identification of biochemical markers for determining the embryogenic potency would be of great help for efficient plant regeneration (Kumar 2011).

Dioscorea is an important medicinal plant belonging to the family Dioscoreaceae which comprises of 600 species and is divided into 23 sections based on characteristics such as stem twining, leaf morphology, inflorescence, seed wings, bulbil formation, tuber morphology and chemical constituent in genus *Dioscorea* (Dahlgren *et al.* 1985). Based on their relative economic importance, there are two unique groups of species (Ammirato 1985, Van Stadin and Fowlds 1992). The first group comprises cultivated species (yams or true yams) while the second group consists of wild species, rich in secondary metabolites having huge medicinal values.

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Dioscorea alata L. a cultivated species commonly known as greater yam and ratalu in Hindi, is a rich source of starch, albuminoids, fat, fibres and P_2O_5. It is a prickly climber with puberulous or pubescent (sometimes villous), stem twining to the left with alternate leaves (Akahori 1965). In India, it is cultivated in Rajasthan, Bihar, Kerala, Assam, West Bengal and Odisha. Underground tubers (yams) are the source of phytochemicals used to treat health ailments such as inflammation, joint pain, diabetes, infection and dysmenorrhea. Analysis of various cellular metabolites during organ differentiation in callus provides a reasonable and promising approach towards an understanding of the biochemical basis of organogenesis (organ development/ plant regeneration) (Kumar *et al.* 2013). In spite of considerable achievements in genetic transformation, least is known about plant cell differentiation in cultures as well as the underlying molecular basis of *in vitro* differentiation (Goyal *et al.* 2009). Little is known about the intervening biochemical events occurring in the cultured cells undergoing organogenesis. Therefore elucidation of biochemical changes, enzymatic changes and protein profile changes accompanying differentiation, will be really helpful in deciphering the underlying mechanisms (Singh *et al.* 2006). The present experiment was aimed to understand the changes in metabolites, enzyme activities and protein profile pattern that occur during organogenesis in *Dioscorea alata*.

MATERIALS AND METHODS

The plant material (tubers) of *Dioscorea alata* was procured from Central Plantation Crops Research Institute, Kassaragod (Kerala) through National Bureau of Plant Genetic Resources (NBPGR), New Delhi. The explants for callus culture were obtained from mature plants grown in the Botanical Garden of the Department of Botany and Plant Physiology, CCS Haryana Agricultural University, Hisar. Murashige and Skoog's medium (Murashige and Skoog 1962) was used as basal medium with 3% sucrose, myo-inositol (100 mg w/v) and 0.8% agar. The leaf, internode, node and tuber explants obtained from field grown plants were cut into small pieces (1 cm) and immediately inoculated, aseptically in the flasks containing MS basal medium with different concentrations and combinations of plant growth regulators for callus initiation. The cultures were kept under photoperiod (2000 lux) of 16 h light at 26 ± 2°C. The high growth rate callus selected on the basis of weight of fresh and dry calli were subcultured on MS media supplemented with different combinations and concentrations of growth regulators for root and shoot differentiation respectively. Various metabolites (starch, total soluble sugars, reducing sugars, total soluble proteins, total phenols and free amino acids) and enzymes (α-amylase, acid invertase, acid protease, peroxidase and acid phosphatase) activities were assayed as well as protein profile resolution mapping was done during root and shoot differentiation from callus in *Dioscorea alata* as following: (1) Control- undifferentiated callus before keeping on differentiating medium. (2) One part of the same callus used for control was kept on root differentiation medium [MS + 2.0 μM NAA + 0.5 μM IBA] and samplings were done at 4th, 8th, 12th and 16th day intervals, respectively. (3) The other part of the same callus used for control was kept on shoot differentiation medium [MS + 2.0 μM BAP + 0.5 μM NAA] and samplings were done at 4th, 8th, 12th and 16th day intervals, respectively

Extraction of metabolites was done by the modified method of Barnett and Naylor (1966). Dry callus (100 mg) was homogenized in 80% ethanol (v/v) and centrifuged for 10 min at 10000 g. The extraction procedure was repeated three times with the residue. The supernatants were pooled and the final volume was made to 5 ml with ethanol and used for estimation of total soluble sugars (Yemm and Willis 1954), reducing sugars (Honda *et al*. 1982), free amino acids (Yemm and Cocking 1955) and total phenols (Amorim *et al.* 1977). The pellet was hydrolyzed with 4 ml of chilled 0.2 N $HClO_4$ at 4°C for 24 h. The hydrolysate was centrifuged at 5000 rpm for 15 min and the supernatant was used for starch estimation (Hassid and Neufeld 1964). Total soluble proteins in the callus were extracted in 0.1 M Tris-HCl (pH 7.5) by using pre-chilled pestle and mortar. The homogenate obtained was centrifuged at 10000 g for 15 min at 4°C and total soluble proteins in the supernatant were estimated using the method of Bradford (1976).

One gram of fresh callus tissue was hand homogenised in pre-chilled pestle and mortar using 0.1 M Tris-HCl (pH 7.5) buffer containing 0.25 mM EDTA, 2.5 mM Cystein-HCl and 0.1% PVP. The homogenate was centrifuged at 10000 rpm for 15 min at 4°C. The supernatant was used for estimating the activities of various enzymes, viz. α-amylase, acid-invertase, acid-protease, peroxidase and acid-phosphatase using the methods of Shuster and Gifford (1962), Summer (1935), Beevers (1968), Seevers *et al*. (1971) and Jones (1969), respectively.

The 25 μl of crude protein extract, containing 50 μg of protein extract was transferred to an equal volume of Laemmli's 2X sample buffer (0.5 M Tris-HCl, pH 6.8) containing 20% glycerol, 4% SDS, 0.5% bromophenol blue (w/v) and 10% β-mercaptoethanol and heated at 100°C for 3 min and cooled. Electrophoresis was carried out by the method of Laemmli (1970). The cooled samples were then loaded on to a SDS-discontinuous gel system with a 0.1 mm thick stacking gel of 4% polyacrylamide in Tris-HCl buffer (pH 6.8) and a resolving gel of 10% polyacrylamide in Tris-HCl buffer (pH 8.8). The gels were run at 15mA in the stacking gel and 25mA in the resolving gel. After electrophoresis, gels were fixed and stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) methanol with 7% glacial acetic acid (v/v) and then destained in 10% methanol (v/v) with 7.5% glacial acetic acid (v/v). After destaining, the gels were stored at 7% glacial acetic acid (v/v).

All the experiments were repeated at least thrice, using

8-10 replicates (flasks) each containing three explants. The data were analyzed using completely randomized design and the significance was tested at 5% level of critical difference (OPSTAT software, CCS HAU).

RESULTS AND DISCUSSION

Among different explants, viz. leaf, node, internode and tuber used for callus induction and growth, node explants were found to be the best for induction and growth of callus. Data presented in Table 1 show that NAA (2.0 and 2.5 µM) either alone or in combination with IBA (0.25 and 0.5 µM) induced good callus growth even up to 4 weeks after inoculation.

For further growth the callus derived from nodal explants of *Dioscorea alata* was subcultured for 4-weeks on the same media. Depending on the weight of fresh and dry callus (Table 2), the best growth was observed on MS medium supplemented with NAA (2.5 µM). Accordingly, this combination was selected for callus maintenance, growth, differentiation of roots/shoots and to identify biochemicals markers (metabolites estimation, enzyme analysis) and protein profiles.

Differentiation of roots and shoots

Organized growth of callus cultures leading to root and shoot differentiation is preceded by changes at the cellular and subcellular levels. An early biochemical marker for the identification of embryogenic potency would therefore be of great help for efficient plant regeneration. Analysis of various cellular metabolites during organ differentiation in callus provides a reasonable understanding of the biochemical basis of organ development (Kumar 2011). Many investigations have been made to unravel the physiological changes taking place during organogenesis in callus cultures. It has been shown that morphogenetic pattern is associated with specific biochemical changes that can be used as markers. In *D. alata*, callus formed on MS medium supplemented with 2.5 µM NAA from nodal explant was shifted for root and shoot differentiation. Calli raised on MS + NAA (2.5 µM) were shifted to MS medium containing NAA (2.0 µM) and IBA (0.5 µM) for root differentiation. The visual appearance of root formation was observed after 12th day of subculture (Table 3). The presence of NAA in the medium enhanced good callus growth while IBA supported the differentiation of roots. However, NAA (2.5 µM) along with 2, 4-D (2.0 µM), induced good growth of callus as well as root and shoot formation within 28 and 31 days after inoculation, respectively. Regeneration of shoot was obtained on MS medium supplemented with BAP (2.0 µM) and NAA (0.5 µM), while green patches were formed before the appearance of shoot formation (Kumar *et al.* 2013). The visual appearance of shoots was observed after 12th day of subculture (Table 3). BAP (2.0 µM) initially supported growth of callus before shoot differentiation characterized by the accumulation of metabolites from 0 to 8th day. These results are supported by the findings of Patel and Shah (2009) in callus cultures of *Stevia rebaudiana*.

Table 1 *In vitro* response of nodal explants for callus induction in *D. alata* (on MS medium supplemented with different growth regulators after 4-weeks of inoculation)

Medium + growth regulators (µM)	Callus induction		
	% explant callusing	Days for callus induction	Visual growth of callus after 4-weeks*
Control	0		
2,4-D (0.5)	0		
(1.0)	15	13	+ (Yellow, fragile)
(1.5)	25	13	+ (Yellow, fragile)
(2.0)	0		+ (Yellow, fragile)
NAA (0.15)	0		
(0.5)	0		
(1.0)	25	10	++ (Brown, compact)
(1.5)	67	10	++ (Brown, compact)
(2.0)	75	10	+++ (Brown, compact)
(2.5)	100	8	+++ (Pinkish brown, compact)
(3.0)	0		
(4.0)	0		
IBA (1.0)	0		
(2.0)	0		
NAA (1.0) + IBA (0.5)	33	24	++ (Brown, fragile)
NAA (2.0) + IBA (0.25)	50	38	+++ (Brown, compact)
NAA (2.0) + IBA (0.5)	66	38	+++ (Brown, compact)
NAA (2.0) + IBA (1.0)	33	38	++ (Brown, compact)
NAA (2.0) + IBA (2.0)	15	38	+ (Brown, compact)
NAA (1.0) + BAP (0.5)	0		
NAA (1.0) + BAP (1.0)	0		
NAA (2.0) + BAP (0.5)	15	37	+ (Green, fragile)
NAA (2.0) + BAP (1.0)	0		
NAA (2.0) + BAP (2.0)	0		
NAA (2.5) + BAP (1.0)	0		
NAA (5.0) + BAP (1.0)	0		
IBA (0.1) + BAP (2.0)	0		
IBA (0.2) + BAP (2.0)	0		

*– No callus, + Poor callus, ++ Moderate callus, +++ Good callus

Table 2 High growth rates obtained on the basis of weight of fresh and dry of 4-weeks old calli derived from nodal explants of *Dioscorea alata* (Callus growth on various concentrations of NAA alone or in combination with IBA supplemented to MS medium)

Medium + Growth regulators (μM)	Weight of callus (g)	
	Fresh weight	Dry weight
MS + NAA (2.0)	3.225±0.059	0.239±0.019
MS + NAA (2.5)	3.45±0.107	0.432±0.019
MS + NAA (2.0)+ IBA (0.25)	3.211±0.082	0.272±0.032
MS + NAA (2.0)+ IBA (0.5)	3.121±0.248	0.227±0.039

Biochemical analysis (metabolites and enzyme activities) during shoot and root differentiation

It was found that starch content in callus increased upto 8th day which declined during the processes of root and shoot differentiation and again increased after shoot initiation (Fig 1 and 2). The decrease in starch content could either be due decreased activity of synthesizing enzymes or increased activity of hydrolyzing enzymes. On the other hand, the content of total soluble sugars showed similar trends of decrease and increase during both root as well as shoot differentiation (Fig 1 and 2). The content of reducing sugars increased in callus gradually upto root initiation and subsequently declined during root formation (Fig 1). However, the content of reducing sugars increased upto shoot differentiation and declined with the visual appearance of shoots (Fig 2). The depletion depicts the utilization of sugars during differentiation process (Goyal *et al.* 2009, Singh *et al.* 2009, Kumar and Goyal 2010, Tyagi and Swarnkar 1995, Chatrath *et al.* 1996). The activity of α-amylase was initially low upto root and shoot differentiation (Fig 3 and 4).

Higher α-amylase activity in the present study during root and shoot induction indicated faster starch degradation. Energy requirement for cell differentiation is quite high (Thorpe and Meier 1972) and hence the greater amylase activity during root and shoot differentiation in callus. Singh *et al.* (2009), Yadav *et al.* (1995), Abe *et al.* (1996) and Singh *et al*. (2011) also suggested that this energy is made

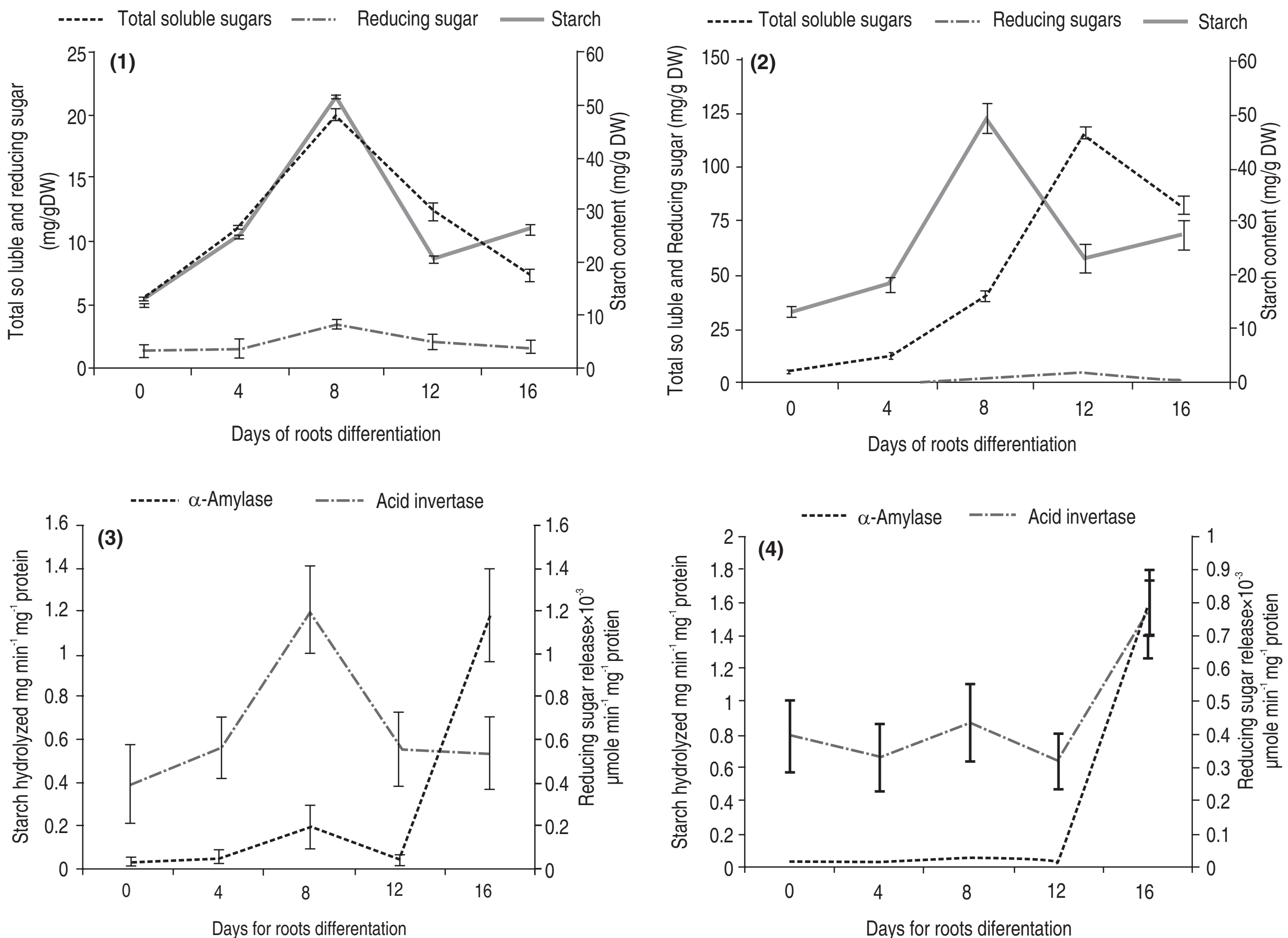

Fig 1-4 The content of soluble sugar, reducing sugars and starch in *D. alata* callus prior to inoculation (0 day), (Fig 1) on rooting medium MS + 2.0 μM NAA + 0.5 μM IBA (4th, 8th, 12th and 16th day); (Fig 2) on shooting medium MS + 2.0 μM BAP + 0.5 μM NAA (4th, 8th, 12th and 16th day) after inoculation (Bars represent SE of the means of three replicates). Changes in the activity of α-amylase and acid invertase in *D. alata* callus prior to inoculation (0 day), (Fig 3) on rooting medium MS + 2.0 μM NAA + 0.5 μM IBA (4th, 8th, 12th and 16th day); (Fig 4) on shooting medium MS + 2.0 μM BAP + 0.5 μM NAA (4th, 8th, 12th and 16th day) after inoculation (Bars represent SE of the means of three replicates).

Table 3 Effect of growth regulators supplemented to MS medium on nodal callus from *D. alata* for root-shoot differentiation (observations recorded upto 6-weeks after inoculation)

Medium + growth regulator (μM)	Visual growth of callus after 4-weeks*	Colour and Texture of callus	Differentiation (Days required)
Control	—	Brown, compact	
NAA (0.5)	+	Black, compact	
(1.0)	+	Black, compact	
(2.0)	++	Brown, compact	
(2.5)	+++	Pinkish brown, compact	
(3.0)	++	Brown, compact	
NAA (2.0) + IBA (0.1)	+	Brown, compact	
NAA (2.0) + IBA (0.5)	+++	Pinkish brown, compact	Roots (15)
NAA (2.0) + IBA (1.0)	++	Brown, compact	Roots (34)
NAA (2.5) + 2, 4-D (0.5)	+	Brown, compact	
NAA (2.5) + 2, 4-D (1.0)	++	Brown, compact	
NAA (2.5) + 2, 4-D (1.5)	++	Brown, compact	
NAA (2.5) + 2, 4-D (2.0)	+++	Brown, compact	Roots (28) & Shoots (31)
NAA (2.5) + 2, 4-D (2.5)	++	Brown, compact	
NAA (2.5) + 2, 4-D (3.0)	++	Brown, compact	
BAP (0.5) + NAA (2.5)	++	Green, compact	
BAP (1.0) + NAA (2.5)	+++	Greenish brown, compact	
BAP (1.5) + NAA (1.5)	+	Black, fragile	
BAP (1.5) + NAA (2.0)	++	Brown, compact	
BAP (2.0) + NAA (0.5)	+++	Pinkish brown, compact	Shoots (16)
BAP (2.0) + NAA (1.0)	+++	Pinkish brown, compact	
BAP (2.0) + NAA (1.5)	++	Green, fragile	Abnormal shoots (34)
BAP (2.0) + NAA (2.0)	+	Black, compact	
BAP (2.0) + IBA (0.2)	+	Green, fragile	Shoots (42)
BAP (2.0) + IBA (1.0)	++	Green, fragile	Shoots (36)

*– No callus, + Poor callus, ++ Moderate callus, +++ Good callus

available through the utilization of starch. This is evidenced by the negative correlation between high α-amylase activity and starch content to sustain higher metabolic activity during cell differentiation. As cell differentiation is a high energy requiring process, the observed decrease in the starch content and increase in total soluble sugars during differentiation would seem to be necessary for mobilization of starch reserves to carbohydrates. Similar trend was also reported in *Chlorophytum borivilianum* (Goyal *et al.* 2009), *Medicago arborea* (Martin *et al.* 2000), *Zamia furfuracea* (Dominic and Joseph 2004), *Tylophora indica* (Singh *et al.* 2009) and *Cardiospermum halicacabum* (Kumar and Goyal 2010, Jeyaseelan and Rao 2005).

The activity of enzyme acid invertase increased upto 8th day but declined during subsequent stages of root differentiation in callus (Fig 3). A similar trend was noted during shoot differentiation as the enzyme activity initially increased and then declined. As the acid invertase is involved in sucrose uptake and removal of sugars from the vacuole, the decrease in acid invertase activity would have direct correlation with differentiation. This is in confirmity with the findings of Singh *et al.* (2009), Kumar and Goyal (2010) and Cuadrado *et al.* (2001). The hydrolysis of sucrose to glucose and fructose is followed by their phosphorylation to hexose monophosphates (Rees 1988). Carbohydrate metabolism in plants may be indirectly controlled by the availability of inorganic phosphate which is regulated by activity of acid phosphatase. Cierezko and Barbachowska (2000) observed that phosphate deficiency in leaves and roots of bean caused accumulation of glucose, sucrose and starch and increase in the activities of invertases, sucrose synthase and sucrose phosphate synthase. However, in the present study, acid phosphatase activity increased up to 8th day followed by a sharp decrease during root and shoot differentiation stages. These results are in agreement with the findings of Kumar and Maherchandani (1988) in *Nicotiana tabacum* and Panigrahi *et al.* (2007) in *Asteracantha longifolia*, relating to reduced acid phosphatase activity during root and shoot formation.

It was observed that total soluble proteins in calli decreased continuously during root and shoot differentiation than control callus in *D. alata*. The free amino acids content increased continuously till the process of root differentiation but decreased with the appearance of roots. In contrast, the free amino acid content decreased in shoot differentiating calli and increased later on in *D. alata*. Similar results were reported by Singh *et al*. (2006) and Jeyaseelan and Rao (2005). The activity of enzyme acid protease exhibited the similar trend to those of TSPs. In root and shoot differentiating calli, the activity of acid protease increased before and after initiation but declined from 12th day onwards. Higher proteolytic activity indicates high rates of degradation of pre-existing storage and other proteins required during differentiation. Dave and Batra (1995) observed high protease activity during somatic embryo formation in *Cuminum* sp. and Yadav *et al.* (1995) during shoots formation in *Nicotiana* sp. and Kumar and Goyal

(2010) in *Cardiospermum halicacabum.* This might be due to depletion of certain amino acids/polypeptides required to initiate root and shoot formation (Dominic and Joseph 2004, Tyagi and Swarnkar 1995, Panigrahi *et al.* 2007).

Phenol content showed a gradual decline during shoot differentiation in callus. However, in root differentiation, phenol content increased till root initiation stage but decreased during root emergence. In brief, gradual increases in phenol content were observed before and after callus. Phenols participate in the formation of cross linking of cell wall constituents which is catalyzed by peroxidase (Goyal *et al.* 2009, Tyagi and Swarnkar 1995, Mader and Fussel 1982, Ozyigit 2008). These authors suggested that enzyme peroxidase regulates lignin formation via oxidative polymerization of hydroxylated cinnamyl alcohols as evident from lignin deposition during the final phase of *in vitro* root differentiation (Miller *et al.* 1985). Peroxidase activity, influenced by phytohormones, seems to be important in controlling the processes of auxin degradation and lignification which affect callus growth and differentiation. The peroxidase activity decreased in differentiating calli as the process continued till the visual appearance of roots and shoots. Similar results were reported by Singh *et al.* (2009), Kumar and Goyal (2010) and Jeyaseelan and Rao (2005).

Protein profiling through SDS-PAGE

Resolution of the polypeptides by SDS-PAGE (10%) exhibited several differences during root and shoot differentiation from calli. In *D. alata*, the number of polypeptide bands resolved in control callus was 11 which remained same until 4th day of callusing on root differentiation medium. Three new polypeptide bands (25.56, 24.35 and 18.2 kDa) appeared in 8 days old calli and the subsequent disappearance of 2 polypeptide bands (89.13 and 27.43 kDa) indicated that root initiation occurred between 8th to 12th days (Fig 5A). Similarly, calli kept on shooting medium showed the maximum 12 bands on 8th day (i.e. green patch initiation stage). On 12th day, disappearance of two polypeptide bands (36.3 and 16.98 kDa) was observed suggesting that shoot initiation started between 8 to 12 days. Further, one polypeptide of 69.13 kDa size disappeared on 16th day (Fig 5B).

The comparison of root and shoot differentiating calli in *D. alata* showed the presence of four root specific bands (MW 25.56, 24.35, 19.13 and 18.2 kDa) in root differentiating callus and 3 specific bands (53.7, 25.12 and 19.13 kDa) in shoot differentiating callus. Synthesis of specific polypeptides of 56 kDa size in *Arachis hypogaea* (Venkatachalam *et al.* 1997), 61.7 kDa in *Chlorophytum borivilianum* (Goyal *et al.* 2009) and of 85.1 kDa in *Cardiospermum halicacabum* (Kumar and Goyal 2010) has been reported during rhizogenesis and polypeptide bands of 97 kDa in *Arachis hypogaea* specific to shoot formation (Venkatachalam *et al.* 1997), 75 kDa in *Centella asiatica* (Agrawal and Subhan 2003), 117.5 and 112.2 kDa in *Chlorophytum borivilianum* (Goyal *et al.* 2009) and 102.3 and 36.31 kDa in *Cardiospermum halicacabum* (Kumar and Goyal 2010) have already been reported in

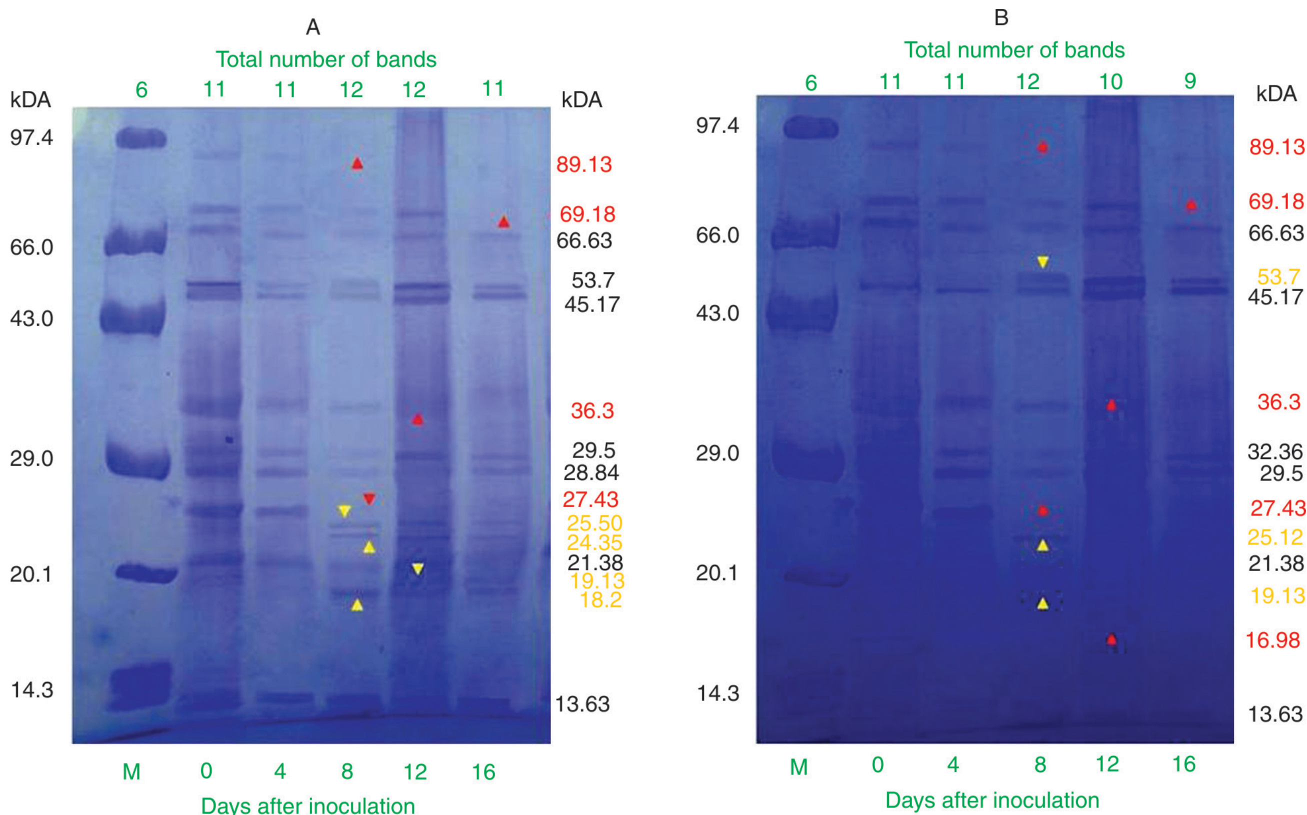

Fig 5 Protein profile in callus of *Dioscorea alata* through SDS-PAGE (10%) prior to inoculation (0 day) (A) on rooting medium (4th, 8th, 12th and 16th day) and (B) on shooting medium (4th, 8th, 12th and 16th day). (M, Molecular weight of protein marker)

litreature. In conclusion, it appears that expression of root/shoot specific polypeptides may be used as markers to characterize differentiation pathways and to augment the selection of regenerating potential callus for rapid *in vitro* propagation in *D. alata*.

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