RESEARCH ARTICLE

Direct shoot organogenesis from leaf explants of *Populus deltoides* and changes in selected enzymatic activities

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Abstract The direct shoot organogenesis was achieved from leaf explant of two commercially important clones of *Populus deltoides* on MS medium enriched with 15 mg/l adenine sulphate, 5 mg/l Ascorbic acid, 250 mg/l (NH₄)₂SO₄ (referred to as PD1 medium) supplemented with 2.5 µM each of 6-benzylaminopurine and indole-3-acetic acid. Higher shoot organogenic potential was recorded from the explants of clone ‘G48’ as compared to clone ‘L34’. The age of leaf explant also affected the shoot organogenic potential, and maximum shoot organogenesis was recorded in case of 5th leaf from the top of microshoot. Histological studies revealed altered cell division resulting in the formation of meristematic pockets after 5 days of culture, these meristematic pockets grew into dome protuberances by 10th day. Organized shoots were visible after 15 days of culture. A clear three phases of shoot organogenesis viz induction (0–4 days), initiation and organization (4–10 days) and growth (11–16 days onwards) were observed. Marked variation in the activity of enzymes such as catalase, peroxidase, polyphenol oxidase and acid phosphatase was observed during these phases. The activity of these enzymes was found to increase in cultures grown on the medium resulting in shoot organogenesis during shoot development (after 7 days of culture).

Keywords Enzyme activity · Explant maturity · Micropropagation · Organogenesis · Plant growth regulators

Introduction

Shoot organogenesis is an important aspect of efficient in vitro plant regeneration. It is a prerequisite to undertake any trait specific genetic modification based on biotechnological approaches (Aggarwal et al. 2011, 2013). Further direct shoot organogenesis is a preferred mode of regeneration over indirect shoot organogenesis as this is reported to have higher efficiency for regeneration of non-chimeric lines (Jehan et al. 1994; Kumar 1996; Kumar and Chandra 2009). The importance of shoot organogenesis can be easily understood by the volume of work done on this aspect in many plant species including *Populus* spp. (Winton 1968; Wolter 1968; Son and Hall 1990; Chaturvedi et al. 2004; Thakur et al. 2005). A few reports are also available on direct shoot organogenesis of *Populus* spp (Rutledge and Douglas 1988; Sellmer et al. 1989; Coleman and Ernst 1990; Noel et al. 2002; Yadav et al. 2009). Most of these studies focus on the role of plant growth regulators (PGRs) on shoot organogenesis (Panigrahi et al. 2007; Vatankhah et al. 2010). There are only a few studies investigating ontogenic events of shoot organogenesis vis-à-vis changes in important enzymes and other biochemical parameters (Brown and Thorpe 1986; Sujatha et al. 2000). Changes in various enzymatic activities have been highlighted as an indicator of various morphogenetic events in many plant species (Kay and Basile 1986; Sujatha et al. 2000; Panigrahi et al. 2007; Vatankhah et al. 2010; Goel et al. 2018). These studies were focussed on the role of various antioxidant enzymes during organogenesis.

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However, the close perusal of literature could not reveal any report investigating the change in activities of important enzymes vis-à-vis ontogenic events of shoot organogenesis. Such studies are likely to provide some information regarding the relation of shoot organogenesis and activities of these enzymes. The changes in enzymatic activity are reported to influence shoot organogenesis from leaf explants of *P. deltoides* (Brown and Thorpe 1986). A thorough understanding of the process of direct shoot organogenesis in *P. deltoides* is important for genetic manipulations, as the potential of explants for the regeneration of shoots/somatic embryos is reported to decrease significantly following *Agrobacterium* infection on selection cum regeneration medium (Aggarwal et al. 2010). Therefore, the present study was focussed to optimize direct shoot organogenesis protocol from leaf explants of *Populus deltoides* and to study the histology of shoot organogenesis and correlate different stages with the activity of selected enzymes.

Materials and methods

Plant material, glassware and chemicals

The shoot cultures of *Populus deltoides* clone, 'G48', 'L34' available at plant tissue culture facility of TIFAC-CORE, TIET, Patiala, India were used for this study. Leaf explants were collected from 21 days old microshoots cultured on MS medium (Murashige and Skoog 1962) enriched with 15 mg/l adenine sulphate, 5 mg/l ascorbic acid, 250 mg/l $(\text{NH}_4)_2\text{SO}_4$ (referred to as PD1 medium: this medium was selected based on performance of cultures on this medium for micropropagation). Unless otherwise mentioned, all chemicals including Plant Growth Regulators (PGRs) were purchased from HiMedia Labs (Mumbai, India). The medium pH was adjusted to 5.8 before autoclaving at 121 °C, 15 psi for 20 min. All tissue culture experiments were conducted in 300 ml culture bottles (Kasablanka Ltd, Mumbai) having 50 ml medium. The incubation temperature of cultures was kept at 25 ± 2 °C with a relative humidity of 50–60% at a photoperiod of 16-h with a light intensity of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ using cool white fluorescent tube lights (Crompton Greaves Ltd, Mumbai).

Shoot organogenesis

Shoot organogenesis from leaf explants of two ('G48', 'L34') clones of *P. deltoides* was attempted on PD1 medium variously supplemented with 6-benzyl adenine (BAP; 0–12.5 μM) and indole-3-acetic acid (IAA; 0–12.5 μM). Care was taken while inoculating the leaves to keep the adaxial surface to touch the medium. Subsequently, the

effect of age of the leaf explant (3rd–7th leaf from top) on shoot organogenic potential was evaluated on PD1 medium supplemented with 2.5 μM each of BAP and IAA (Table 1: the combination induced maximum shoot organogenesis).

Histological analysis

Explants cultured for different periods (0–21 days) were fixed in FAA (formaldehyde: acetic acid: 50% ethanol; 5:5:90; v/v). After a week of fixing, samples were dehydrated by passing through a series of tertiary butyl alcohol (TBA) and then embedded in paraffin wax (Kumar et al. 1999). A rotary microtome (RMT30, Radical Instruments, Ambala Cantt., India) was used to cut tissue sections of 10 μm . Staining was carried out with toluidine blue using protocol of Sharma and Millam (2004), and mounted in dibutylphthalate polystyrene xylene (D.P.X.). The observations were carried out using a compound microscope (Nikon Instruments Inc., Japan).

Extraction of total soluble proteins

The enzyme activities were studied during the shoot organogenesis. The composite samples (1.0 g FW each) were fixed after 0, 3, 5, 7, 10, 14, 21 days of inoculation. These were extracted as describe earlier (Goel et al. 2018). In short, samples were homogenized in phosphate buffer (50 mM, pH 6.8, containing 100 mM sucrose and 1 g l^{-1} cysteine hydrochloride, 200 mg l^{-1} PVP) using mortar pestle. The homogenates were centrifuged ($12,000 \times g$, 45 min, 4 °C) and supernatants were collected. Total volume was made up to 10 ml with extraction buffer. These extracts were used for the estimation of total soluble proteins and to assay activities of peroxidase, catalase and polyphenol oxidase. For assaying the activities of alkaline and acid phosphatase, proteins were extracted in chilled citrate buffer (50 mM, pH 5.3).

Estimation of total soluble proteins

A portion of each extract (5.0 ml) was used for the estimation of total soluble proteins. Extracts were mixed with an equal volume of 20% (w/v) of trichloroacetic acid and proteins were allowed to precipitate for 4 h at 4 °C. The samples were then centrifuged ($1000 \times g$, 30 min at 4 °C). Pellets were collected and dissolved in 2.0 ml of 1 N NaOH. Proteins were estimated following the method of Lowry et al. (1951) using standard curve prepared taking known concentrations of bovine serum albumin.

Peroxidase (POX): POX assay was carried out according to McEwen (1971). The reaction mixture consisted of 2.75 ml phosphate buffer (50 mM, pH 6.8), 0.1 ml O-dianisidine solution (0.05% w/v in methanol),

Table 1 The effect of different concentration of Indole-3-acetic acid and 6-benzyl aminopurine (BAP) on shoots organogenesis from leaf explants of *P. deltooides* on PD1 medium

IAA (μ M)	BAP (μ M)	% Explant showing regeneration		No. of shoots per explant	
		Mean $\pm$ SE		Mean $\pm$ S.E	
		G48	L34	G48	L34
0	0	0** ^{A,i}	0** ^{A,j}	0** ^{A,e}	0** ^{A,h}
1.0	1.0	22 $\pm$ 1.05 ^{A,i}	12 $\pm$ 0.32 ^{A,j}	0.82 $\pm$ 0.05 ^{A,de}	0.40 $\pm$ 0.02 ^{B,gh}
1.0	2.5	54 $\pm$ 1.75 ^{A,f}	16 $\pm$ 0.52 ^{B,g}	2.56 $\pm$ 0.12 ^{A,c}	1.94 $\pm$ 0.36 ^{A,cd}
1.0	5.0	60 $\pm$ 2.02 ^{A,d}	18 $\pm$ 0.95 ^{B,f}	4.26 $\pm$ 0.62 ^{A,b}	2.60 $\pm$ 0.3 ^{A,bc}
1.0	12.5	5 $\pm$ 0.26 ^{A,c}	22 $\pm$ 0.52 ^{A,e}	4.55 $\pm$ 0.98 ^{A,b}	2.08 $\pm$ 0.20 ^{B,cd}
2.5	0	4 $\pm$ 0.57 ^{B,ghi}	3 $\pm$ 0.16 ^{A,i}	2.06 $\pm$ 0.25 ^{A,cd}	0.60 $\pm$ 0.09 ^{B,fgh}
2.5	1.0	60 $\pm$ 2.32 ^{A,ghi}	40 $\pm$ 0.01 ^{B,c}	5.20 $\pm$ 0.36 ^{A,ab}	3.06 $\pm$ 0.35 ^{B,b}
2.5	2.5	88 $\pm$ 3.89 ^{A,c}	76 $\pm$ 1.02 ^{B,a}	6.02 $\pm$ 0.21 ^{A,a}	4.7 $\pm$ 0.68 ^{A,a}
2.5	5.0	79 $\pm$ 3.02 ^{A,b}	70 $\pm$ 1.36 ^{B,b}	5.03 $\pm$ 0.63 ^{A,ab}	1.90 $\pm$ 0.34 ^{B,cde}
2.5	12.5	34 $\pm$ 2.33 ^{A,e}	29 $\pm$ 0.96 ^{A,d}	1.96 $\pm$ 0.21 ^{A,cd}	1.10 $\pm$ 0.01 ^{B,efg}
5.0	0	0* ^{A,i}	0* ^{A,j}	0* ^{A,e}	0* ^{A,h}
5.0	1	54 $\pm$ 2.04 ^{A,d}	40 $\pm$ 0.83 ^{B,c}	3.03 $\pm$ 0.60 ^{A,c}	1.30 $\pm$ 0.09 ^{B,df}
5.0	2.5	65 $\pm$ 1.63 ^{A,c}	42 $\pm$ 1.29 ^{B,c}	3.00 $\pm$ 0.11 ^{A,c}	2.06 $\pm$ 0.25 ^{B,cd}
5.0	12.5	10 $\pm$ 0.99 ^{A,g}	4 $\pm$ 0.25 ^{B,hi}	1.03 $\pm$ 0.13 ^{A,dc}	0.50 $\pm$ 0.05 ^{B,fgh}
12.5	0	0* ^{A,i}	0* ^{A,j}	0* ^{A,e}	0* ^{A,h}
12.5	1	8 $\pm$ 0.12 ^{A,gh}	6 $\pm$ 0.82 ^{B,h}	0.85 $\pm$ 0.29 ^{A,de}	0.40 $\pm$ 0.31 ^{A,gh}
12.5	2.5	7 $\pm$ 0.72 ^{A,gh}	2 $\pm$ 0.11 ^{B,ij}	0.30 $\pm$ 0.32 ^{A,e}	0.20 $\pm$ 0.12 ^{A,h}
12.5	5.0	5 $\pm$ 0.77 ^{A,gh}	0* ^{A,j}	0.13 $\pm$ 0.4 ^{A,e}	0* ^{A,h}
12.5	12.5	2 $\pm$ 0.15 ^{A,gh}	0* ^{A,j}	0.10 $\pm$ 0.27 ^{A,e}	0* ^{A,h}

Leaf explants were taken from 21 days old microshoots of *P. deltooides*. Each experiment consist of 5 replicates with 10 explants per culture vessel

Data were analysed by ANOVA and mean values followed by same lowercase letters within columns are not significant at $P < 0.05$ by DMRT

Means between clones were compared by unpaired t test and those which are significant at $P < 0.05$ are marked with different uppercase letters

0.05 ml H_2O_2 (1% v/v) and reaction was started with the addition of 0.1 ml enzyme extract. Change in absorbance was recorded at 470 nm for 3 min at an interval of 15 s against blank consisting of reaction mixture lacking enzyme extract. Calculations of peroxidase activity were done using the molar extinction coefficient and expressed as units/mg protein.

Catalase assay: Catalase activity was determined according to Aebi (1984). The reaction mixture consisted of 2.85 ml phosphate buffer (50 mM, pH 6.8), 0.05 ml of 30% (v/v) H_2O_2 and 0.1 ml enzyme extract. The specific activity of catalase was determined by a change in absorbance for 3 min at 240 nm and expressed in terms of μ moles of H_2O_2 decomposed per minute. The reaction mixture lacking enzyme extract was used as blank.

Polyphenol oxidase (PPO): The activity of PPO was assayed using pyrogallol as substrate (Kar and Mishra 1976). To 2.0 ml of phosphate buffer (100 mM, pH 6.0), 0.5 ml pyrogallol solution (0.01 M) was added and the reaction was initiated with the addition of 0.5 ml enzyme extract. Change in absorbance was recorded for 3 min (in

an interval of 30 s) at 420 nm against blank consisting of reaction mixture lacking enzyme extract. Specific enzyme activity was calculated by taking one unit enzyme activity required for the degradation of 1 μ mol of pyrogallol $min^{-1} mg^{-1}$ protein.

Phosphatases: The activity of phosphatases (both acid and basic) was assayed according to Sadasivam and Manickam (1992). Tissue samples (composite 1.0 g FW each, fixed after 0, 3, 5, 7, 10, 14, 21 days after incubation) were homogenized in cold (4 °C) citrate buffer (50 mM, pH 5.3) and centrifuged (1000 $\times$ g, 10 min 4 °C) and supernatant was used as enzyme extract. To 3.0 ml of substrate solution (0.4 mM EDTA, 0.45 mM citric acid and 0.05% *p*-nitrophenyl phosphate) pre incubated for 5 min at 37 °C, 0.5 ml of enzyme extract was added and incubation was continued. The reaction was terminated with the addition of 0.1 M NaOH after 15 min (0 min in case of blank). Absorbance was recorded at 405 nm using UV–vis spectrophotometer (Shimadzu, India). For the determination of alkaline phosphatase activity, the extraction buffer consisted of 50 mM glycine–NaOH and

substrate solution consisted of 0.2% (w/v) *p*-nitrophenyl phosphate in 0.42 M NaOH and rest all the procedure was same as in the case of acid phosphate. Enzyme activity was expressed in terms of nmoles of para nitrophenyl phosphate released min^{-1} .

Experimental design and statistical analysis

Unless otherwise stated, all experiments were conducted in triplicate and repeated thrice. Composite sampling (samples were withdrawn from many culture vessels and mixed before taking final sample) were assayed from three different experiments for determining the activity of various enzymes. Data were analyzed by two-way ANOVA. Graphs were plotted using GraphPad Prism version 5 (GraphPad Software, USA).

Results

Shoot organogenesis

The leaf explants showed increase in size following 3 days of culture, protrusions were observed within 5–10 days of culture (Fig. 1a). These further developed into shoots within 10–15 days (Fig. 1b–d). Amongst various combinations of BAP and IAA (0–12.5 μM), highest shoot organogenesis was observed on PD1 medium supplemented with 2.5 μM each of BAP and IAA (Table 1). Higher shoot organogenic potential was observed from explants of clone ‘G48’ as compared to clone ‘L34’ and therefore, for all further investigations related to enzymatic analyses, clone ‘G48’ was used.

Effect of leaf age

The response of leaf segments to organogenesis was also found to be influenced by the leaf explant age. Using fifth leaf from the top (14th–16th day) of microshoots of clone ‘G48’, maximum organogenesis was observed, with 80% of the explants resulting in the formation of shoots and a mean of 8.03 shoots/explant were observed. Therefore, 5th leaf from the top was used as explant for further experiments related to enzymatic analyses. It was also found that organogenic response in very young as well as old leaf was found to decrease (Fig. 2).

Histological studies

Histological studies revealed initial signs of altered cell division after 5 days of culture from epidermal and hypodermal layers of leaf explants cultured on PD1 medium supplemented with 2.5 μM each of BAP and IAA (Fig. 1e).

These centers (showing altered cell division) further grew into dome protuberances by 10th day (Fig. 1f). These dome protuberances further grew and appeared as organized shoots after 15 days of culture (Fig. 1g). The fully grown shoots can be clearly seen by 21st day of culture from the explant surface (Fig. 1d) and have vascular connection with the explant (Fig. 1h).

Enzyme activity

Changes in enzymatic activities were studied following culture of leaf explants on shoot induction medium (PD1 containing 2.5 μM each of BAP and IAA) vis-à-vis non shoot induction media (basal PD1 medium and PD1 supplemented with 12.5 μM each of BAP and IAA). The activity of catalase enzyme was found to increase significantly after 7 days of culture on medium showing shoot induction and it continued to increase up to 21 days of culture (Fig. 3a). On the other two media combinations (with no shoot organogenesis), this increase in the activity of catalase was not observed (Fig. 3a). The peroxidase and polyphenol activities showed similar trends; on medium showing shoot organogenesis (Fig. 3b, c), the activities of these enzymes increased after 5 days of culture and was maximum after 14 days, thereafter a sharp decline was observed from 14th to 21st day, a period coinciding with the shoot growth phase (Fig. 3b, c). A significant change in the activities of these enzymes was recorded with time in these cultures (Table 2). The activities of these enzymes was found to be maximum after 7 days of culture on medium containing 12.5 μM each of BAP and IAA. There was no alteration in the activity of these enzymes on PD1 medium lacking PGRs (Fig. 3b, c).

The activity of alkaline phosphatase did not show significant alterations following different periods of culture on PD1 medium and shoot induction medium (Fig. 4a). However, alkaline phosphatase activity increased following culture on medium containing 12.5 μM each of BAP and IAA and peaked after 14 days of culture. Acid phosphatase activity showed a significant increase after 7th day of culture on the shoot induction medium and was maximum after 21st day (Fig. 4b, Table 2), whereas on basal PD1 medium, the change in acid phosphatase activity was not recorded (Fig. 4b).

Discussion

In vitro shoot organogenesis (both direct and indirect) is important for microporpagation as well as genetic manipulations of the plants (Aggarwal et al. 2011, 2013). For an efficient plant genetic manipulation, a robust shoot organogenesis protocol is a prerequisite (Aggarwal et al.

Fig. 1 Shoot regeneration from leaf explant. **a** Leaf explant cultured on MS medium enriched with 15 mg/l Ads, 5 mg/l Ascorbic acid, 250 mg/l $(\text{NH}_4)_2\text{SO}_4$ (PD1 medium) supplemented with 2.5 μM each BAP and IAA, **b** the dome protuberances proliferation from leaf explant after 10 days of culture, **c** A clear growing shoot like structure from leaf explant after 15 days of culture, **d** A well developed shoot growing from leaf explant after 21 days of culture. **e–h** Histological studies during shoot organogenesis. **e** a section of the leaf explant showing beginning of altered cell division (arrow) after 5 days of culture; **f** a section of well-developed dome protuberances from the leaf explant after 10 days of culture representing formation of Shoot primordia, **g** a section of well developed shoot buds after 14 days of culture, **h** a section of shoot growing from explant after 20 days of culture. Bars = 100 μm

2010). In vitro propagation has emerged as a boon for economically important trees (Vinoth and Ravindhursan 2013). Although, there is a remarkable progress in achieving in vitro shoot regeneration in many plants, yet there are problems with in vitro regeneration of woody plant species (Giri et al. 2004). Moreover, *Populus* spp. have been reported to be recalcitrant for shoot organogenesis when explant source is mature plants and there is need to optimize the regeneration protocol for each species (Noël et al. 2002; Cui et al. 2014; Jiang et al. 2015; Kwon et al. 2015). In this study, direct shoot organogenesis was achieved from the leaf explants taken from microshoots of *P. deltoides* clone ‘G48’ and clone ‘L34’ on PD1 medium (MS medium enriched with 15 mg/l adenine sulphate,

5 mg/l ascorbic acid and 250 mg/l $(\text{NH}_4)_2\text{SO}_4$) supplemented with 2.5 μM each of BAP and IAA. Earlier, direct shoot organogenesis has been reported from the petiole explants of the clone ‘G48’ (Thakur et al. 2012). The present study reporting shoot organogenesis from leaf explants taken from the microshoots of two commercially important clones namely, ‘G48’ and clone ‘L34’ seems to be novel information. The direct shoot organogenesis achieved in this study has the advantage over indirect organogenesis as the regenerated plants are likely to be genetically stable and true-to-type (Thakur et al. 2012). In this study, maximum shoot organogenesis was achieved on PD1 medium supplemented with 2.5 μM each of BAP and IAA. Earlier, the concentration of PGRs in the medium has

Fig. 2 The effect of age of leaf (leaf number from top of shoot) on shoot organogenesis in clone 'G48' on PD1 medium supplemented with 2.5 μ M each of BAP and IAA

been shown to be an important factor for shoot regeneration in many plants from different explants (Van Staden et al. 2008; Kumar et al. 2010; Aggarwal et al. 2010). However, among the clones, high shoot organogenic potential was observed in clone 'G48' and the clonal differences in regeneration response has been well known in *Populus* spp. (Jiang et al. 2015; Kwon et al. 2015).

In the present study, the maturity of leaflets also has a significant effect on shoot organogenesis (Fig. 2). In both the clones, maximum response with respect to percent explants showing shoot organogenesis and number of shoots per explant were significantly higher in case of the fifth leaf from the top. The effect of leaf age on shoot organogenesis has earlier been reported in *Eucalyptus tereticornis* (Aggarwal et al. 2010), apple (Yepes and Aldwinckle 1994) and *Anthurium andraeanum* (Martin et al. 2003). Thus, selecting the explant of appropriate maturity level could be helpful for shoot organogenesis.

In the present study, altered cell division was observed in the epidermal and hypodermal cells of leaf explants after 5 days of culture resulting in the formation of meristematic pockets (Fig. 1). These meristemoids further grew into dome protuberances by 10 days (Fig. 1f). Further, within 15 days these protuberances grew into shoots. Thus, a clear course of shoot organogenesis in *P. deltoides* was worked out indicating the phases such as shoot induction (0–4 days), shoot initiation and organization (5–10 days) and shoot growth (10–15 days onwards). These results are line with the earlier reports on histology of shoot organogenesis in *Populus trichocarpa* $\times$ *deltoides* (Jehan et al. 1994) and *Bacopa monnieri* (Sarkar and Jha 2017). Earlier, de Klerk et al. (1997) has also recorded three phases during shoot organogenesis and somatic embryogenesis in plants.

The changes in enzyme activities that were studied correlated with the various histological stages of shoot organogenesis and a clear trend of expression of these enzymes was observed in explant tissues on medium

Fig. 3 The activities of **a** catalase, **b** peroxidase and **c** polyphenol oxidase in the explant tissues cultured on different media combination for different periods of culture (0–21 days) during shoot organogenesis from leaf explants of *P. deltoides* clone 'G48'

showing shoot organogenesis (supplemented with 2.5 μ M each of BAP and IAA) and other combinations with no shoot organogenesis. Earlier such expression patterns of the antioxidant enzyme have been reported in *Crocus sativa* (Vatankhah et al. 2010). The activity of various enzymes and protein content increased following culture of explants as compared to 0 days (beginning of experiment). Dougall (1962) and Syono (1965) also suggest the important role of protein synthesis during organogenesis.

Table 2 Two way ANOVA table of effect of different hormone concentration and culture period on peroxidase activity, catalase activity, polyphenol oxidase, acid phosphatase and alkaline phosphatase activities

Source	df	Sum of squares	Mean square	F	P
Peroxidase activity					
Interaction between concentrations and culture periods	6	67,360	11,230	339.1	0.0001
Between concentrations	2	112,100	56,070	1694	0.0001
Between culture period	12	87,080	7257	219.2	0.0001
Error	42	1390	33.11		
Catalase activity					
Interaction between concentrations and culture periods	6	4468	744.6	91.52	0.0001
Between concentrations	2	3544	1722	217.8	0.0001
Between culture period	12	4934	411.1	50.53	0.0001
Error	42	341.7	80,136		
Polyphenol oxidase					
Interaction between concentrations and culture periods	6	466.0	77.66	60.58	0.0001
Between concentrations	2	2051	1026	800.1	0.0001
Between culture period	12	1194	99.47	77.59	0.0001
Error	42	53.84	1.282		
Acid phosphatase					
Interaction between concentrations and culture periods	6	14.48	2.413	879.8	0.0001
Between concentrations	2	46.12	23.06	8410	0.0001
Between culture period	12	77.23	6.436	2347	0.0001
Error	42	0.1493	0.003554		
Alkaline phosphatase					
Interaction between concentrations and culture periods	6	1.609	0.2682	31.79	0.0001
Between concentrations	2	9.387	4.694	556.3	0.0001
Between culture period	12	3.471	0.2892	34.28	0.0001
Error	42	0.3544	0.008437		

Catalase enzyme is known to inactivate hydrogen peroxide to oxygen and water (Mhamdi et al. 2010). Catalase activity increased after 7 days which may be correlated with higher production of hydrogen peroxide during shoot induction and organization. In the present study, a sharp increase in the peroxidase activity was also observed during shoot induction and organization phase. The increase of peroxidase activity and alteration of its isozymes patterns have been correlated with adventitious shoot formation and other ontogenic events (Kochba et al. 1977; Thorpe and Gaspar 1978; Kay and Basile 1986). The findings of present study corroborates with these reports. The increased activity of peroxidase was associated with various physiological processes, such as lignification, auxin catabolism, wound healing and defense mechanism (Hiraga et al. 2001). Patel and Thorpe (1984) observed that the accumulation of proteins during organogenesis was due to higher expression of peroxidase, acid phosphatase and ATPase in organ forming regions. The lower activity of peroxidase was correlated with decreased cell division (de

Marco and Roubelakis-Angelakis 1996). Availability of inorganic phosphate indirectly controls carbohydrate metabolism in plants, which in turn is reported to regulate the activity of acid phosphatase. A sharp increase in acid-phosphatase during shoot differentiation in this study has a positive correlation with shoot organization and growth, a phase of higher metabolic requirement. Earlier, maximum acid-phosphatase activity has also been observed during similar phases of shoot organogenesis in cultured hypocotyls of *Jatropha integerrima* (Sujatha et al. 2000). Alkaline phosphatase activity in the cultures on shoot induction medium remained constant and lower indicating not much role of this enzyme.

Conclusion

The efficient direct shoot organogenesis protocol was established from the leaf explants of two commercially important clones of *Populus deltoides*. Amongst clones,

Fig. 4 The activities of **a** alkaline phosphatase, **b** acid phosphatase in the explant tissues cultured on different media combination for different periods of culture (0–21 days) during shoot organogenesis from leaf explants of *P. deltoides* clone ‘G48’

higher shoot organogenic potential was observed in clone ‘G48’. The leaf maturity also affected shoot organogenic potential of the leaf explants and maximum percent of leaf explants resulted in shoot organogenesis when 5th leaf was used. Different stages of shoot organogenesis were marked with higher activity of the enzymes such as catalase, peroxidase, polyphenol oxidase and acid phosphatase.

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