Plant Growth Regul (2009) 59:199–205
DOI 10.1007/s10725-009-9404-x

ORIGINAL PAPER

The effect of thidiazuron level on in vitro regeneration type and peroxidase profile in *Eucalyptus microtheca* F. Muell

Morteza Shabannejad Mamaghani · Mohammad Hassan Assareh · Mansoor Omidi · Mohammad Matinizadeh · Abbas Ghamari-Zare · Shokofeh Shahrzad · Massih Forootan

Received: 30 April 2009 / Accepted: 3 August 2009 / Published online: 19 August 2009

Abstract The green twigs of 1-year-old *Eucalyptus microtheca* F. Muell seedlings were cultured on modified MS medium, supplemented with α-naphthalene acetic acid (NAA) and kinetin (Kin) hormones at 12 different concentrations. After 4 weeks, the combination of 1 mg l^{-1} NAA + 1 mg l^{-1} Kin induced the highest number of axillary shoots. Meanwhile, embryogenic calli were observed in media containing 4 mg l^{-1} NAA + 0.5 mg l^{-1} Kin, without any regeneration. The hormone treatments were followed by subculturing the twigs in different levels of thidiazuron (TDZ). The combination of 1 mg l^{-1} NAA + 1 mg l^{-1} Kin together with 0.01 mg l^{-1} TDZ resulted in an increase of direct shoot, while higher amounts of TDZ led to adventitious shoot induction. Somatic embryogenesis was observed in the treatment containing 0.01 mg l^{-1} TDZ + 4 mg l^{-1} NAA + 0.5 mg l^{-1} Kin. The peroxidase (POD) band patterns in regenerated plantlets were investigated in order to determine the effect of different levels of TDZ on loci synthesis. A dimer locus, a tetramer locus and two epigenetic bands (a new band for NAA + Kin and the other for TDZ) were observed in the POD profiles. In case of low (0.01 mg l^{-1} and 0.1 mg l^{-1}) levels of TDZ, one heterozygote allele was disappeared from dimer locus, while at higher TDZ levels, the dimer locus lost its stability and tetramer locus showed a high activity. Thus, POD allele patterns seems to be a feasible marker for different types of regeneration.

Keywords Adventitious shoot · Direct shoot · *Eucalyptus* · Peroxidase · Somatic embryogenesis · Thidiazuron

Abbreviations

Kin	Kinetin
NAA	α-Naphthalene acetic acid
POD	Peroxidase
TDZ	Thidiazuron

Introduction

Approximately 16 million ha of lands in Iran are located in the desert marginal area. *Eucalyptus* spp. is found to be an adaptable species which can be utilized in stabilizing the eroding lands (Assareh 1998). Over 700 species, subspecies and natural hybrids from *Eucalyptus* genus have been identified (Watt et al. 1999; Pinto et al. 2002). Among those, *Eucalyptus microtheca* F. Muell is native to Australia and has been introduced to the southern subtropical

M. Shabannejad Mamaghani (✉)
Department of Plant Breeding, Faculty of Agriculture and Natural Resources, Islamic Azad University, Karaj Branch, P.O. Box 31485–313, Karaj, Tehran, Iran
e-mail: m.shabannejad@gmail.com

M. H. Assareh · A. Ghamari-Zare · S. Shahrzad · M. Forootan
Department of Biotechnology, Research Institute of Forests and Rangelands, P.O. Box 13185–116, Tehran, Iran

M. Omidi
Department of Plant Breeding, Faculty of Agriculture, University of Tehran, P.O. Box 31587–11167, Karaj, Tehran, Iran

M. Matinizadeh
Forest Research Division, Research Institute of Forests and Rangelands, P.O. Box 13185–116, Tehran, Iran

regions of Iran and has been adapted to the ecological conditions of this area. The species is not desirable for wood industry; however, improvements are possible using breeding techniques (Assareh 1998). Maintaining elite genotypes in this species is very difficult due to open pollination. The in vitro tissue culture enables us to control the essential factors in differentiation, regeneration, and proliferation. Moreover, it can be a proper tool to manipulate the environmental factors and plant material homogeneity (Morabito et al. 1994).

Differentiation is a multi-stage procedure, at the end of which the regeneration is carried out in forms of direct and indirect shooting, as well as somatic embryogenesis. In vitro regeneration is necessary for asexual proliferation of elite genotypes and gene transformation (Tang and Newton 2005). Explants derived from direct regeneration are genetically more stable than the ones passing through callus formation stage (Tang and Newton 2005). Adventitious shoot in *E. camaldulensis* (Muralidharan and Mascarenhas 1987) and *E. gunnii* (Herve et al. 2001), direct shoot in *E. tereticornis* (Sankara Rao 1988) and somatic embryogenesis in *E. dunnii* and *E. grandis* (Watt et al. 1999), *E. nitens* (Bandyopadhyay and Hamill 2000), *E. globulus* (Pinto et al. 2002) and *E. sargentii* and *E. camaldulensis* (Assareh 1998) has been reported. Comparing these methods indicates that (a) Juvenile explants have great potential for regeneration in *Eucalyptus*, (b) Using NAA with a proper cytokinin, will increase regeneration, (c) Using casein and glutamine amino acids or Thidiazuron (TDZ) for direct shoot, adventitious shoot and somatic embryogenesis induction in *Eucalyptus* is common and (d) A post-treatment with low level of hormone seems necessary for the growth of embryos.

Peroxidase (POD) has been reported to be used as a marker for rhizogenesis, organogenesis and embryogenesis (Coppens and Dewitte 1990; Gaspar et al. 1992; Preetha et al. 1995). This enzyme has high activity as a marker during stressful conditions (Ezaki et al. 1996). POD activity will increase during callus production, it has been reported to produce specific bands in embryogenic calli (Xiaoli et al. 1992) and also in different stages of regeneration (Rout et al. 1999; Panigrahi et al. 2007). On the other hand, significant change of POD and catalase (CAT) activity as a marker in pine direct shoot by TDZ has been observed (Tang and Newton 2005). Also, the effect of abscisic acid (ABA) on POD promoter has been recognized (Klotz and Lagrimini 1996) but no hormone has been identified to show a special band profile in different levels.

Peroxidase is affected by environment, genotype and their interaction. In this study, the objective was to study the effect of TDZ on regeneration efficiency. In vitro culture was exploited to eliminate environmental effects, and then variation in POD profiles at different TDZ levels was investigated in different types of regeneration.

Materials and methods

Investigation of the regeneration potential

Leaf discs, leafstalks, and green twigs without wood tissue, were separated as explants from a seedling plant which was kept in greenhouse for 1 year. For surface sterilization, the explants were immersed in sterilized water containing some drops of Tween for 15 min. Then they were rinsed with distilled water and soaked in mercuric chloride solution (0.01%) for 30 s. The explants were finally rinsed three times with sterile distilled water (5 min each time under laminar air flow cabinet). Modified MS (Murashige and Skoog 1962) media, with KNO_3 and NH_4NO_3 at half-strength level (autoclaved for 20 min in 120°C) were used for regeneration. NAA and Kin were used in four (0.5, 1.0, 2.0, and 4.0 mg l^{-1}) and two concentrations (0.0, 0.5, and 1.0 mg l^{-1}), respectively, as in Table 1. After surface sterilization, the explants were cut into 1–1.5 cm long segments, cultured in regeneration media (culture) for 4 weeks, maintained in photoperiod of 16 h of light (27°C) and 8 h of darkness (19°C). Four replications containing six explants were used for each treatment.

Optimizing the process of differentiation

Based on the results of previous experiments, the green twigs were found as the best explants and were separated from the same seedling plant for the rest of the study. "Materials and methods" described above were used for sterilizing the explants, preparing the culture medium and maintaining culture conditions. Four different concentrations of TDZ plant growth regulator (0.0, 0.01, 0.1, 0.5, and 1.0 mg l^{-1}) were added to the culture media that had shown superior performance (Table 2). The leaves of plantlets were used as samples for the rest of the study 30 days after culture.

POD quality survey

Crude extraction was conducted using Ebermann and Stich method (1982). Polyacrylamide gel electrophoresis was carried out using the method of Ebermann and Stich (1982). 0.1 g of fresh leaf was homogenized in 0.3 ml extraction buffer (1.2 g l^{-1} Tris, 3.8 g l^{-1} Borax, 3.6 g l^{-1} Chloride sodium, 2 g l^{-1} Ascorbic acid, 50 g l^{-1} polyethylene glycol 2000, 2 g l^{-1} Na_2EDTA, pH = 7.5) and was kept for 48 h in 4°C. The samples were centrifuged at 6,000*g*, for 15 min in 4°C. Supernatant was used for electrophoresis with 12% separating gel (120 g l^{-1} acrylamide, 2 g l^{-1} bisacrylamide, 45.6 g l^{-1} Tris, pH = 8.3), which was loaded in the following conditions 5% staking gel (50 g l^{-1} acrylamide, 8.33 g l^{-1} bisacrylamide,

Table 1 Callus production in *E. microtheca* explants using combinations of NAA and Kin levels

Treatments	Additives	Explant response	Leaf	Leafstalk	Twig
H_1	NAA 0.5 (mg l^{-1}) + Kin 0.0 (mg l^{-1})	Callus production	−	−	−
		Embryogenic callus	−	−	−
H_2	NAA 0.5 (mg l^{-1}) + Kin 0.5 (mg l^{-1})	Callus production	+	−	−
		Embryogenic callus	No	−	−
H_3	NAA 0.5 (mg l^{-1}) + Kin1.0 (mg l^{-1})	Callus production	−	−	−
		Embryogenic callus	−	−	−
H_4	NAA 1.0 (mg l^{-1}) + Kin 0.0 (mg l^{-1})	Callus production	+	+	−
		Embryogenic callus	No	No	−
H_5	NAA 1.0 (mg l^{-1}) + Kin 0.5 (mg l^{-1})	Callus production	+	−	+
		Embryogenic callus	No	−	No
H_6	NAA 1.0 (mg l^{-1}) + Kin 1.0 (mg l^{-1})	Callus production	−	−	+
		Embryogenic callus	−	−	Yes
H_7	NAA 2.0 (mg l^{-1}) + Kin 0.0 (mg l^{-1})	Callus production	+	−	−
		Embryogenic callus	No	−	−
H_8	NAA 2.0 (mg l^{-1}) + Kin 0.5 (mg l^{-1})	Callus production	++	+	+
		Embryogenic callus	No	No	No
H_9	NAA 2.0 (mg l^{-1}) + Kin 1.0 (mg l^{-1})	Callus production	+	+++	−
		Embryogenic callus	No	No	−
H_{10}	NAA 4.0 (mg l^{-1}) + Kin 0.0 (mg l^{-1})	Callus production	−	−	−
		Embryogenic callus	−	−	−
H_{11}	NAA 4.0 (mg l^{-1}) + Kin 0.5 (mg l^{-1})	Callus production	+++	++	++++
		Embryogenic callus	No	No	Yes
H_{12}	NAA 4.0 (mg l^{-1}) + Kin 1.0 (mg l^{-1})	Callus production	++	−	++
		Embryogenic callus	No	−	No

Traits were registered after 4 weeks. Callus production, embryogenic callus, (−) indicates not observing, (+) bad quality, (++) poor, (+++) good and (++++) the best quality, (Yes) presence of embryogenic calli, (No) absence embryogenic calli. Data recording method from Pinto et al. (2002)

15 g l^{-1} Tris and 8.4 g l^{-1} citric acid) and electrolyte buffer (7.2 g l^{-1} glycine 1.5 g l^{-1} Tris) with 300 V and 100 mA during 4 h. 50 µl from each sample was loaded into the wells. Staining was done by 1,800 µl carbazole solution, (0.04 g carbazole, 1,200 µl absolute ethanol and 600 µl acetic acid) with 750 µl 3% H_2O_2 and 100 ml acetate buffer (50 mM, pH = 4.5) for 30 min. Then the gel was rinsed by distilled water and image was taken using a Sony digital camera. POD profile changes were carried out using two independent gels inclusive two repeats for maternal plant and minimum three independent repeats for other treatments.

Results

Tissue culture

In the first experiments, 7 days after culture initiation, callus production appeared from the cutting edge of the explants. Low level of NAA produced small calli, which showed increase in leaf disc and leafstalk explants in media with higher NAA levels. No embryogenic callus was observed (Table 1). Friable and embryogenic calli were formed in H_6 and H_{11} treatments on the green twig explants (Table 1). Twig explants in H_6 treatment produced very little callus (light green) on which microshoots appeared (Fig. 1a). Thirty days after the differentiation, when different amounts of TDZ were added to H_6 treatment, the regeneration rate was 10% in B_1 and 74% in B_2 (Table 2). In B_2 treatment callus production was not observed (Fig. 1b) and regeneration was of direct shoot type (Fig. 1c). There was no regeneration in treatment containing 0.1 mg l^{-1} TDZ. In B_4 treatment, callus production was derived from cambium in twig explants and pre-embryonic white cells were appeared on green calli. In treatment containing 0.5 mg l^{-1} TDZ pre-embryonic cells passed dedifferentiation course (Fig. 2a–d) and regeneration was of adventitious shoot type, regeneration in B_4 was 14% (Fig. 2e, f). Using B_5 treatment, the calli became compact and hard, and the rate of adventitious shoot was reduced to 2%. Yellow-colored embryogenic calli

Table 2 Optimizing differentiation by TDZ in *E. microtheca* explants

Treatments	Best treatments of first stage with different concentration of TDZ hormone	No. of explants tested	Average no. of responsive explants (%)	Embryogenic callus production	Regeneration type	Total no. of regenerations	Average no. of regenerations (%)
B_1	NAA 1.0 (mg l^{-1}) + Kin 1.0 (mg l^{-1}) + TDZ 0.0 (mg l^{-1})	42	45.23	Yes	Axillary shoot	17	10
B_2	NAA 1.0 (mg l^{-1}) + Kin 1.0 (mg l^{-1}) + TDZ 0.01 (mg l^{-1})	42	88.09	–	Direct shoot	124	74
B_3	NAA 1.0 (mg l^{-1}) + Kin 1.0 (mg l^{-1}) + TDZ 0.1 (mg l^{-1})	42	–	No	–	–	0
B_4	NAA 1.0 (mg l^{-1}) + Kin 1.0 (mg l^{-1}) + TDZ 0.5 (mg l^{-1})	78	23.07	Yes	Adventitious shoot	43	14
B_5	NAA 1.0 (mg l^{-1}) + Kin 1.0 (mg l^{-1}) + TDZ 1.0 (mg l^{-1})	24	12.5	No	Adventitious shoot	2	2
B_6	NAA 4.0 (mg l^{-1}) + Kin 0.5 (mg l^{-1}) + TDZ 0.0 (mg l^{-1})	18	–	Yes	–	–	0
B_7	NAA 4.0 (mg l^{-1}) + Kin 0.5 (mg l^{-1}) + TDZ 0.01 (mg l^{-1})	42	35.71	Yes	Somatic embryogenesis	57	19
B_8	NAA 4.0 (mg l^{-1}) + Kin 0.5 (mg l^{-1}) + TDZ 0.1 (mg l^{-1})	42	80.95	Yes	Somatic embryogenesis	244	81
B_9	NAA 4.0 (mg l^{-1}) + Kin 0.5 (mg l^{-1}) + TDZ 0.5 (mg l^{-1})	30	–	No	–	–	0
B_{10}	NAA 4.0 (mg l^{-1}) + Kin 0.5 (mg l^{-1}) + TDZ 1.0 (mg l^{-1})	24	–	No	–	–	0

Number of explants tested, average number of responsive explants (%), embryogenic callus production, regeneration type, total number of regenerations and average number of regenerations (%) in B_1 and B_{10} treatments were evaluated. (–) indicates not observing, (Yes) presence of embryogenic calli, (No) absence embryogenic calli. Data were recorded 30 days after culture. Data recording method from Pinto et al. (2002)

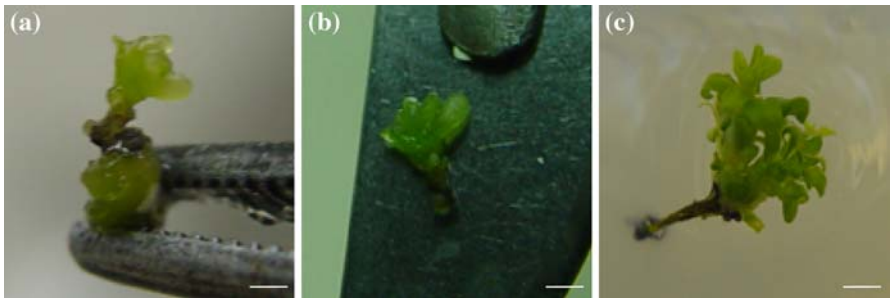

Fig. 1 Trend of callus suppression and direct shoot induction in 1 year old explants of twig *E. microtheca* plant. **a** Axillary shooting in H_6 treatment without using TDZ, callus is seen in small amount (*bar* 0.4 cm). **b** Callus suppression in direct shoot formation using B_2 treatment (*bar* 0.1 cm). **c** Regeneration in form of direct shoot (*bar* 0.3 cm)

showing globular and heart stage embryos were observed in H_{11} treatment (Table 1) but there were no regeneration (Fig. 3a). When 0.01 mg l^{-1} TDZ was added to H_{11} treatment (4 mg l^{-1} NAA and 0.5 mg l^{-1} Kin), there was 19% somatic embryogenesis induction (Fig. 3b). The highest amount of somatic embryogenesis (81%) was in B_8 treatment (Fig. 3c).

POD qualitative tests

Mother plants as controls and plantlets of adventitious origin were compared in qualitative surveys (Fig. 4). In the maternal plant, there was a group of three allele dimers and a group of five allele tetramers (Fig. 4a). There were two new extra bands in the plantlets derived from tissue culture. The first band with lower activity appeared in light molecular area (band 10), using NAA and Kin in all samples (100%). When different amounts of TDZ were added to culture media, 87.5% of regenerated plantlets had one extra band in heavy molecular weight area (band 1). In B_1 treatment, all alleles existed and three, four and nine alleles had more activity (Fig. 4b). In B_2 and B_7 treatments, in plantlets which were regenerated via direct shooting and somatic embryogenesis, allele three was eliminated and the activity of dimer

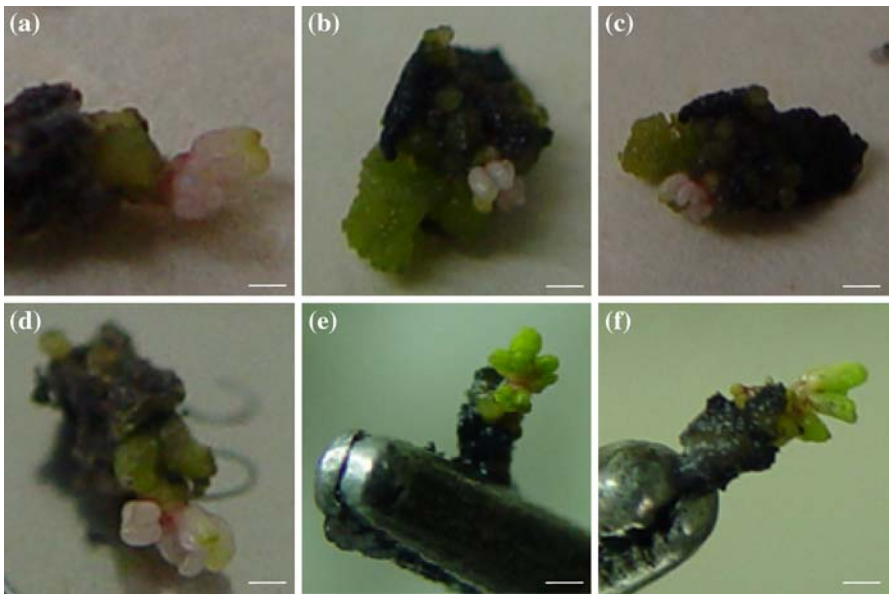

Fig. 2 Regeneration trend in twigs of *E. microtheca* plant using B_4 treatment. **a** Pre-embryonic cells in globular stage (*bar* 0.05 cm). **b** Pre-embryonic cells in heart shape stage (*bar* 0.05 cm). **c** Pre-embryonic cells in while start torpedo stage (*bar* 0.05 cm). **d** Pre-embryonic cells in cotyledon leaves appearance stage and beginning of photosynthesis (*bar* 0.1 cm). **e**,**f** Regeneration in adventitious shoot formation (*bar* 0.4 cm)

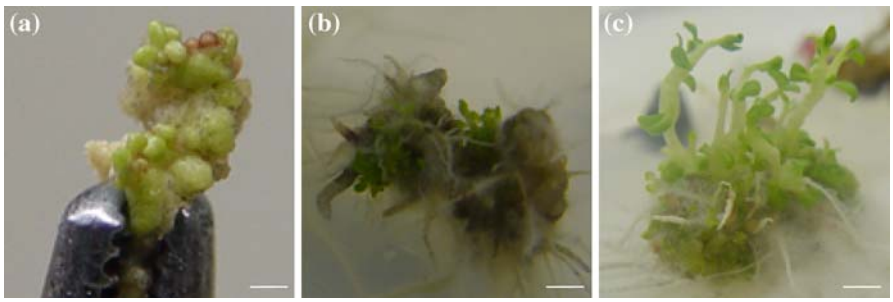

Fig. 3 Somatic embryogenesis induction in 1 year old explants of twig *E. microtheca* plant. **a** Embryogenic friable callus in H_{11} treatment that had heart shape and globular stages but would not make regeneration (*bar* 0.4 cm). **b** Embryogenic calluses resulted from B_7 treatment in which they had a few regeneration (*bar* 0.5 cm). **c** More regeneration in somatic embryogenesis formation using B_8 treatment (*bar* 0.5 cm)

and tetramer alleles locus reduced (Fig. 4c, d). Plantlets derived from somatic embryogenesis in B_8 treatment, had an active tetramer locus, while the activity of dimer locus was similar to treatments B_2 and B_7, namely allele three was disappeared (Fig. 4e). All alleles existed in 50% of B_4 treatment plantlets and the activity of tetramer locus had increased drastically (Fig. 4f). In the other 50% of plantlets, dimer locus had lost its stability and became inactive (Fig. 4g). POD alleles in plantlets derived from adventitious shoot in B_5 treatment showed similar behavior as in B_4 but both locus were highly active (Fig. 4h).

Discussion

In this study, the medium containing 1 mg l^{-1} NAA and 1 mg l^{-1} Kin proved to be the best treatment for axillary shoot regeneration of *E. microtheca* twig explants. The result of this research agrees with report of Sankara Rao (1988) in which uniformity concerning type and concentration of used auxin for direct shoot in *E. tereticornis* was demonstrated.

The twig explants of *E. microtheca* in treatment containing 4 mg l^{-1} NAA along with 0.5 mg l^{-1} Kin showed the most friable embryogenic calli. For induction of somatic embryogenesis in *Eucalyptuses*, 3–5 mg l^{-1} NAA has been used in previous studies (Muralidharan et al. 1989; Pinto et al. 2002).

Adding 0.01 mg l^{-1} TDZ to H_6 treatment resulted in decrease in callus production and regeneration was in direct shoot form. In other words, TDZ in small amount is likely to have suppressive effect on callus production but stimulated regeneration (Shan et al. 2000; Tang and Newton 2005). Visser et al. (1992) showed that benzyl amino purine and TDZ are hormones that can stimulate shoot formation.

Thidiazuron increase in H_6 treatment to 0.5 mg l^{-1} increased callus production and stimulated the formation of

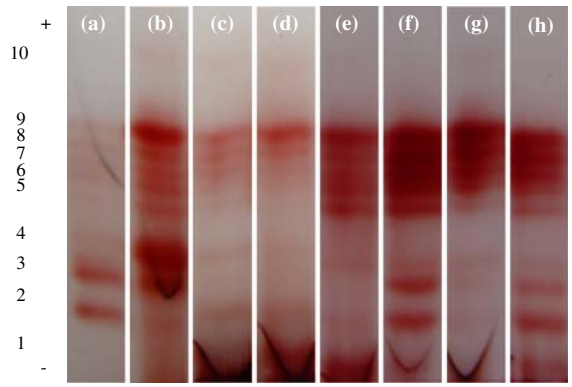

Fig. 4 POD bands resulted from somaclonal plantlets of *E. microtheca*. Maternal plant has been used as a check plant for B_1 treatment plantlets and from B_1 treatment for evaluated other treatments containing TDZ hormone were used. **a** Maternal plant that containing two locus of dimer (alleles 2–4) and tetramer (alleles 5–9). **b** Alleles activity in axillary shooting plantlets of B_1 treatment and show new lower epigenetic band (band 10) in light molecular weight area. **c**,**d** The alleles are resulted from plantlets of B_2 and B_7 treatments (direct shoot and somatic embryogenesis), respectively that was with eliminated of heterozygote allele (allele 3) from dimer locus along with appearance of new epigenetic band (band 1) in heavy molecules weight area. **e** Shows reactivation of tetramer locus and disappearance of heterozygote allele (allele 3) in plantlets resulted from somatic embryogenesis in B_8 treatment. **f**,**g** Show stability and instability in dimer locus also hyperactivity of dimer and tetramer locus in plantlets resulted from treatment B_4 (adventitious shoot). **h** Shows hyperactivity of both locus in plantlets of B_5 treatment. + and − show light and heavy molecules weight area, respectively

adventitious shoots. In this treatment, the existence of compact bulks of white pre-embryonic cells is a possible evidence for a balance between cytokinin and auxin ratio. The presence of auxins, stimulates reproduction of pre-embryonic cells in callus, but has a preventive effect on growth (Nomura and Komamine 1985; Vries et al. 1998; Filonova et al. 2000). So it can be postulated that the combination of cytokinin and auxin has been optimized by adding TDZ. TDZ can highly stimulate and balance endogenous auxin/cytokinin levels (Visser et al. 1992). In the joint of pre-embryonic cells to the callus, phenol accumulation could be observed at the basal of embryos. Phenol accumulation has been reported in different stages of embryogenesis and regeneration (stages) of various species of *Eucalyptus* (Muralidharan and Mascarenhas 1987). Phenol affects the cell wall structure and plant growth regulators have role on their accumulation (Fry 1979).

In B_7 and B_8 treatments, that contain 0.01 and 0.1 mg l^{-1} TDZ, respectively, the regeneration took place through somatic embryo formation. Embryogenic calli in these treatments, contrary to H_{11} treatment, have formed many roots.

Peroxidase bands appearance or disappearance, and their rate of activity are affected by type and amount of hormones, as well as regeneration type (Joersbo et al. 1988). Band 10 is correlated with NAA and Kin, in all samples except the maternal plant, with very low activity. This band can be a marker for in vitro tissue culture or the use of NAA and Kin hormones. Compared to previous samples, band 1 exists whenever TDZ is added to the culture medium. In other words, this band is a marker for the use of TDZ in culture media. Some studies have discussed the impact of auxins and cytokinins on POD profile (Krsnik-Rasol 1991; Hassanian 2004; Panigrahi et al. 2007).

In axillary shoots, POD activity in dimer and tetramer locus, increases remarkably. There was no visible difference between B_2 and B_7 treatments which cause direct shoot and somatic embryogenesis, respectively. POD activity was low in direct shoot of white pine explants using TDZ hormone (Tang and Newton 2005). In B_2 and B_7 treatments the activity of all alleles decreases and heterozygote allele 3 disappears in all plantlets. Allele 3 disappeared in B_8 treatment as well. So it can be concluded that TDZ in small amounts (0.01 and 0.1 mg l^{-1}) may cause inactivity in a heterozygote allele from a dimer locus. This may be due to the synthesis of an inhibitor for POD promoter (Klotz and Lagrimini 1996) or due to mutation (Larkin and Scowcroft 1981). Phytohormones can affect POD promoter (Klotz and Lagrimini 1996). Tetramer locus activity in somatic embryos increased and heterozygote allele from a dimer loci disappeared. POD bands activity pattern in barley embryogenic calli, has been introduced as biochemical marker (Coppens and Dewitte 1990).

There's stability in dimer and tetramer locus in all regeneration types under treatments containing 0.01 and 0.1 mg l^{-1} TDZ. Adventitious shoots initiated when TDZ level exceeds 0.5 mg l^{-1}. This is while Tian et al. (2003) have reported reduction of POD activity in organogenesis. Tang and Newton (2005) observed reduction of POD activity in adventitious shoots for five to 6 weeks and after that they observed POD increase again. This reduction activity can be due to TDZ hormone ability for deleting a POD specific allele, which create instability or prevent activity of one or a few POD locus during regeneration.

Acknowledgments This research was supported by funds from the Research Institute of Forests and Rangelands of Iran.

References

Assareh MH (1998) In vitro culture plant regeneration through organogenesis, somatic embryogenesis and photoautotrophic micropropagation of some *Eucalyptus*. National University of Ireland. Ph.D. Thesis, pp 1–200

Bandyopadhyay S, Hamill J (2000) Ultrastructural studies of somatic embryos of *Eucalyptus nitens* and comparisons with zygotic embryos found in mature seeds. Ann Bot 86(2):237–244

Coppens L, Dewitte D (1990) Esterase and peroxidase zymograms from barley *(hordeum vulgare* L.) callus as a biochemical marker system of embryogenesis and organogenesis. Plant Sci 67:97–105

Ebermann R, Stich K (1982) Peroxidase and amylase isoenzymes in the sapwood and heartwood of trees. Phytochemical 21:2401–2402

Ezaki B, TsugUa S, Matsumoto H (1996) Expression of moderately anionic peroxidase is induced by aluminum treatment in *Tobacco* cells: possible involvement of peroxidase isozymes in aluminium ion stress. Physiol Plant 96:21–28

Filonova L, Bozhkov P, von Arnold S (2000) Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking. J Exp Bot 51(343):249–264

Fry SC (1979) Phenolic components of the primary cell wall and their possible role in the hormonal regulation of growth. Planta 146(3): 343–351

Gaspar T, Kevers C, Hausman JF, Berthon JY, Ripetti V (1992) Practical uses of peroxidase activity as a predictive marker of rooting performance of micropropagated shoots. Agronomie 12(10): 757–765

Hassanian AM (2004) Effect of relatively high concentration of mannitol and sodium chloride on regeneration and gene expression of stress tolerant (*Alhagi Graecorum*) and stress sensitive (*Lycopersicon Esculentum* L.) plant species. Bulg J Plant Physiol 30:19–36

Herve P, Jauneau A, Paques M, Marien JN, Michel Boudet A, Teulieres C (2001) A procedure for shoot organogenesis in vitro from leaves and nodes of an elite *Eucalyptus gunnii* clone: comparative histology. Plant Sci 161(4):645–653

Joersbo M, Andersen JM, Okkels FT, Rajagopal R (1988) Isoperoxidases as markers of somatic embryogenesis in carrot cell suspension cultures. Physiol Plant 76:10–16

Klotz KL, Lagrimini LM (1996) Phytohormone control of the tobacco anionic peroxidase promoter. Plant Mol Biol 31:565–573

Krsnik-Rasol M (1991) Peroxidase as a developmental marker in plant tissue culture. Inter J Dev Biol 35:259–263

Larkin PJ, Scowcroft WR (1981) Somaclonal variation—a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214

Morabito D, Mills D, Prat D, Dizengremel P (1994) Response of clones of *Eucalyptus microtheca* to NaCl in vitro. Tree Physiol 14(2):201–210

Muralidharan EM, Mascarenhas AF (1987) In vitro plantlet formation by organogenesis in *E. camaldulensis* and somatic embryogenesis in *Eucalyptus citriodora*. Plant Cell Rep 6(3):256–259

Muralidharan EM, Gupta PK, Mascarenhas AF (1989) Plantlet production through high frequency somatic embryogenesis in long term cultures of *Eucalyptus citriodora*. Plant Cell Rep 8: 41–43

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with *Tobacco* tissue culture. Physiol Plant 15(3):473–479

Nomura K, Komamine A (1985) Identification and isolation of single cell that produce somatic embryos at a high frequency in a carrot suspension culture. Plant Physiol 79(4):988–991

Panigrahi J, Behera M, Maharana S, Mishra RR (2007) Biomolecular changes during in vitro organogenesis of *Asteracantha longifolia* (L.) nees—a medicinal herb. Indian J Exp Biol 45:911–919

Pinto G, Santos C, Neves L, Araujo C (2002) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Rep 21(3):208–213

Preetha N, Yasodha R, Gurumurthi K (1995) Peroxidase isoenzymes as markers of organogenesis in *Azadirachta indica* Juss. Indian J Plant Physiol 38:92–93

Rout GR, Samantaray S, Das P (1999) Root induction in microshoots of *Simarouba glauca* L. In vitro: peroxidase as a marker for rooting. Silvae Genet 48:14–17

Sankara Rao K (1988) In vitro meristem cloning of *Eucalyptus tereticornis* Sm. Plant Cell Rep 7(7):546–549

Shan X, Li D, Qu R (2000) Thidiazuron promotes in vitro regeneration of wheat and barley. In Vitro Cell Dev Biol Plant 36(3):207–210

Tang W, Newton RJ (2005) Peroxidase and catalase activities are involved in direct adventitious shoot formation induced by thidiazuron in eastern white pine (*Pinus* strobus L.) zygotic embryos. Plant Physiol Biochem 43(8):760–769

Tian M, Gu Q, Zhu M (2003) The involvement of hydrogen peroxide and antioxidant enzymes in the process of shoot organogenesis of strawberry callus. Plant Sci 165:701–707

Visser C, Qureshi JA, Gill R, Saxena PK (1992) Morphoregulatory role of thidiazuron, substitution of auxin and cytokinin requirement for the induction of somatic embryogenesis in *Geranium* hypocotyls cultures. Plant Physiol 99(4):1704–1707

Vries SC, Booji H, Meyerink P, Huisman G, Wild DH, Thomas TL, van Karman A (1998) Acquisition of embryogenic potential in carrot cell suspension culture. Planta 176(2):196–204

Watt MP, Blakeway FC, Termignoni R, Jain SM (1999) Somatic embryogenesis in *Eucalyptus grandis* and *E. dunni*. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 5. Kluwer, UK, pp 63–78

Xiaoli Z, Yang H, Wenjie Y, Ti X (1992) Somatic embryogenesis and analysis of peroxidase in cultured lettuce (*Lactuca Sativa* L.) cotyledons. Ann Bot 69:97–100