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Full Length Research Paper

Microsatellite loci and peroxidase alleles correlation in somaclonal variation of *Eucalyptus microtheca* F. Muell

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The aim of this study was to investigate the correlation between biochemical and molecular markers in *Eucalyptus microtheca* F. Muell. under *in vitro* culture. For this mean, twig-derived explants obtained from *Eucalyptus microtheca* 1-year-old seedling were cultured on modified MS medium, supplemented with different concentrations of NAA, Kin and TDZ. POD (peroxidase) alleles patterns were studied among regenerated plantlets to investigate the effect of TDZ concentration on POD activity. A dimer locus, a tetramer locus and two epigenetic bands were observed. Genome variation among somaclonal plantlets were investigated using microsatellite markers. SSR (Simple Sequence Repeat) markers revealed polymorphism among the studied population. Nonparametric statistical analysis showed significant effect of simple sequence repeats loci on peroxidase alleles. Correlation of two similarity matrix POD and SSRs loci was 0.18 using Mental test. Results showed less stability of dimer locus in different concentrations of TDZ compared to tetramer locus. Tetramer alleles showed more correlation to SSRs than that of dimmer ones.

Key words: Tissue culture, somaclonal variation, peroxidase, simple sequence repeat, *Eucalyptus microtheca.*

INTRODUCTION

Eucalyptus microtheca F. Muell was introduced to Iran and has been adapted to the environmental conditions of this region (Assareh, 1998). The species is not desirable for its trunk shape, but breeding is possible by assessment of new hybrids and selection of superior genotypes (Assareh, 1998). Due to open pollination of this species, conserving the elite families and genotypes is very difficult. That is why *in vitro* propagation enables us to have more control over differentiation, regeneration, and proliferation; it is a proper tool to reduce the effect of environment and obtain a uniform population (Morabito et al., 1994). Organogenesis in *Eucalyptus gunnii* (Herve et al., 2001), direct shoot in *Eucalyptus tereticornis* (Sankara Rao, 1988) and somatic embryogenesis in *E. gunnii*, *Eucalyptus grandis* (Watt et al., 1999) and *Eucalyptus globulus* (Pinto et al., 2002) have been achieved in previous

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Abbreviations: NAA, α-Naphthalene acetic acid; **Kin,** kinetin; **TDZ,** thidiazuron; **POD,** peroxidase; **SSR,** simple sequence repeat.

Table 1. Hormonal treatments used for regeneration of *Eucalyptus microtheca* F. Muell.

Treatments	Hormones (mgl^{-1})			Average number of regeneration (%)
	NAA	Kin	TDZ	
B_1	1	1	0	6
B_2	4	0.5	0.01	20
B_3	4	0.5	0.1	52
B_4	1	1	0.5	19
B_5	1	1	1.0	3

studies.

POD is affected by environmental factors, genotype and their interaction. It is considered to be a marker to recognize organogenesis and embryogenesis (Preetha et al., 1995). On the other hand, the significant changes in POD and CAT (catalase) activities are proved to be an indicator of direct regeneration by TDZ in pine (Tang and Newton, 2005). The rate of POD activity in precotyledonary and cotyledonary stages is estimated to be little; which is similar to that of zygotic embryo stages (Kormutak et al., 2003). Increasing POD activity has been observed in callus initiation and embryonic calli in lettuce (Xiaoli et al., 1992). This enzyme is also involved in completing cell walls, polysaccharide cross-sectional joint and lignin synthesis (Preetha et al., 1995; Fry, 1979; Castillo et al., 1986). Meanwhile, this enzyme is known as a marker against stress (Ezaki et al., 1996).

Polymorphism in the SSRs has shown to be correlated with enzyme activity (Kinoshita and Ishikawa, 2003). In response to the stress, imposed on the POD promoter of generic sequences in tobacco, ABA phytohormone controls the balance of this promoter (Klotz and Lagrimini, 1997). Comparing molecular markers by Liu and Furnier (1993), Rus-Kortekaas et al., (1994), Chen and Sun, (1997) and Russell et al. (1997) demonstrated the correlation between enzyme and molecular markers. The correlation of SSR molecular marker has been compared with the other molecular markers on barley and soya plants (Saghai-Maroof et al., 1994; Powell et al., 1996; Powell and Waugh, 1997), the correlation isoenzymes and SSR molecular marker on *Triticeae* (Sun et al., 1998) has been investigated. The coefficient r = 80% between microsatellite bands and RAPD, using Spearman rank, has been reported by Sun et al. (1997). Moreover, Mental test (1967) showed the correlation between microsatellites and isoenzymes to be 0.164% on *Triticaceae* (Sun et al., 1997). Also Shah et al. (1999) and Byrne et al. (1996) have used the Chi-square test to determine the significant locus. The possible correlation between SSR and POD profiles and the probable effect of TDZ on genomic variation in *E. microtheca* was investigated.

MATERIALS AND METHODS

Plant material

Green twigs without wood tissue were separated from a seedling plant which was kept in greenhouse for one year as explant. The explants were immersed in tap water, containing some drops of tween for fifteen minutes. Then they were rinsed with distilled water and were treated by 0.01% mercuric chloride (w/v) for 30 s. Finally the explants were rinsed by double distilled water (5 min each). MS medium (Murashige and Skoog, 1962), with half-strength KNO_3 and NH_4NO_3 were used for explant culture. According to Table 1, five treatments with different concentrations of plant growth regulators were used in culture media. After surface sterilizing the explants, they were cut into 1 to 1.5 cm segments and put in medium culture. Each treatment had seven replications in which six explants were cultured. They were maintained in 16 h of light (27°C) and 8h of darkness (19°C) conditions for four weeks. After this period of time, the leaves of somaclonal plantlets were sampled for further studies.

POD quality survey

Crude extraction and polyacrylamide gel electrophoresis was carried out using Ebermann and Stich (1982) with PAGE method for 27 somaclonal plantlets that had enough fresh leaf. 0.1 g of fresh leaf was homogenized in 0.3 ml extraction buffer (pH = 7.5) and was kept 48 h in 4°C. The samples were centrifuged in 12000 rpm, for 15 min in 4°C. Supernatant was used for electrophoresis with 12% separating gel (120 gl^{-1} acrylamide, 2 gl^{-1} bisacrylamide, 45.6 gl^{-1} tris, and pH = 8.3 which was adjusted by citric acid), was loaded in the following conditions: 5% staking gel (50 g l^{-1} acrylamide, 8.33 gl^{-1} bisacrylamide, 15 gl^{-1} tris and 8.4 gl^{-1} citric acid) and electrolyte buffer (7.2 gl^{-1} glycine and 1.5 gl^{-1} tris) with 300 V and 100 mA during 4 h. 50 µl from each sample was injected into each lane. In order to stain, 1800 µl carbazole solution, (0.04 g carbazole, 1200 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) were used for 30 min. Then the gel was rinsed in distilled water and an image was made out of it.

Investigation on SSRs changes

DNA extraction was carried out by Doyle and Doyle (1987) method, PCR primers selected from EMBRA06 (Brondani et al., 1998) and EMCRC09 (Steane et al., 2001) (Table 2) for 33 somaclonal plantlets. The components were used according to Table 3 for 25 µl reaction.

Table 2. SSR primers used for *Eucalyptus microtheca* F. Muell analysis.

Primers name	Sequence	Motif	Allele range
EMBRA06-Fwd EMBRA06-Rev	5'-AGAGAATTGCTCTTCATGGA-3' 5'-GAAAAGTCTGCAAAGTCTGC-3'	$(AG)_{19}$	120-170
EMCRC 09-Fwd EMCRC 09-Rev	5'-CTGGGCTGTGCATCTCTGAAA-3' 5'-GACCCGGTCAACTCCTCAAGA-3'	$(TG)_{14}$	286-342

Fwd: Forward; Rev: reverse.

Table 3. PCR components for amplification of *Eucalyptus microtheca* F. Muell.

Components reaction	Concentration of used	Optimize content for one 25 µl PCR reaction
DNA	100 ng	
Buffer PCR	200 mM AMS, 500 mM KCl	2.75
$MgCl_2$	15 mM	2
Taq DNA Polymerase	2 u μl^{-1}	2
dNTP Mix	0.1 mM	2.5
Fwd. primer	0.4 mM	2
Rev. primer	0.4 mM	2

Byrne et al. (1996) method was used for PCR reaction steps for gene amplification. For observing bands, 1.7% agarose gel and for staining, ethidium bromide were used. Recognizing polymorphism was carried out using 6% denatured polyacrylamide gel with silver staining.

Statistical analysis

The outcomes of different experiments were analyzed by the GLM procedure in SAS software. For more clarity of POD bands, zymogram were created using Excel software. Sake markers scoring used 1 (present) and 0 (absent). In all surveys analysis used, 27 somaclonal plantlets were identified by tow markers. Chi-squared test was used to investigate the significant correlation of SSR molecular marker on POD alleles (Shah et al., 1999; Byrne et al., 1996). Meanwhile rank coefficient between these bands was carried out through JMP software, using Spearman nonparametric method (Lanner-Herrera et al., 1996). To get the correlation coefficient between two POD markers and SSR, using NTSYS software, first, two Jaccard's similarity coefficient matrix was estimated and then correlation was calculated using Mental test (1967) (Sun et al., 1998).

RESULTS

Tissue culture

After four weeks, the explants in treatment B_1 showed direct regeneration (Figure 1A). Adding TDZ to media already containing NAA and Kin, the amount of regeneration increased from 6% (B_1), reached to 20% (B_2). The highest amount of regeneration was 52% in B_3. In this treatment, the regeneration was via somatic embryogenesis (Figure 1B). In B_4 there was a drastic increase of callus production, and indirect regeneration peaked at its highest amount, while somatic embryogenesis decreased. The regeneration rate in B_4 was 19% (Figure 1C). In B_5 treatment, calli were compact and hard and as a result, indirect regeneration had a reduction of 3%. After differentiation, all plantlets had normal appearance.

POD quality tests

The quality of POD profile within 27 somaclonal plantlets along with the maternal plant was evaluated (Figures 2 and 3). There was a group of three allele dimer and a group of five allele tetramer, as a mother plant (2-4 and 5-9 alleles) (Figure 4A). There were two new bands in the plantlets which resulted from tissue culture. The first one with lower activity appeared in light molecular area, using NAA and Kin hormones in all samples (100%). When different amounts of TDZ hormone were used to complete the culture medium, 21 plantlets and 42 samples were regenerated. There was another new band in heavy molecules area (band 1, Figures 2 and 3). In B_1 treatment, all alleles existed and 3, 4 and 9 alleles were more active (Figure 4B) but in B_1 and B_3 treatments, allele 3 was deleted and the activity rate of the other alleles decreased relatively (Figure 4C). All alleles existed in 50% of B_4 treatment, and tetramer locus was highly active. In the other 50%, dimer locus activity reduced remarkably (Figures 4D and E). In plantlets generated from

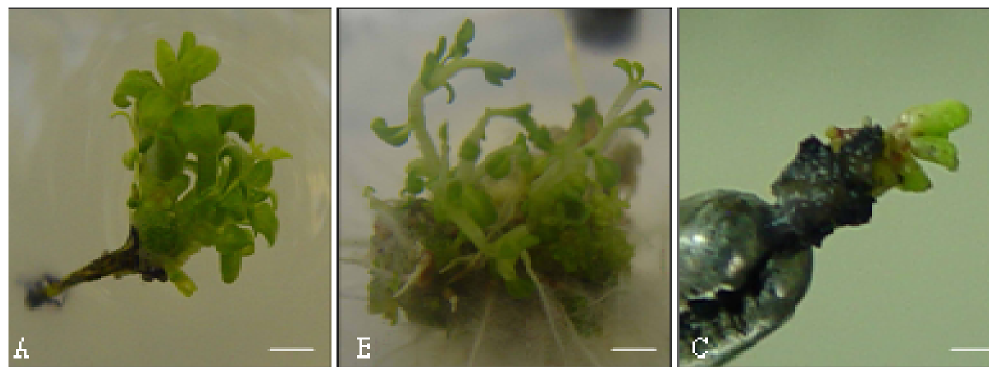

Figure 1. Different regeneration types *in vitro* in *E. microtheca*. A: Direct Shoot in B_1 treatment (bar = 0.4 cm), B: Regeneration from embryogenic callus in B_3 treatment (bar = 0.5cm) and C: Indirect regeneration in B_4 treatment (bar = 0.3 cm).

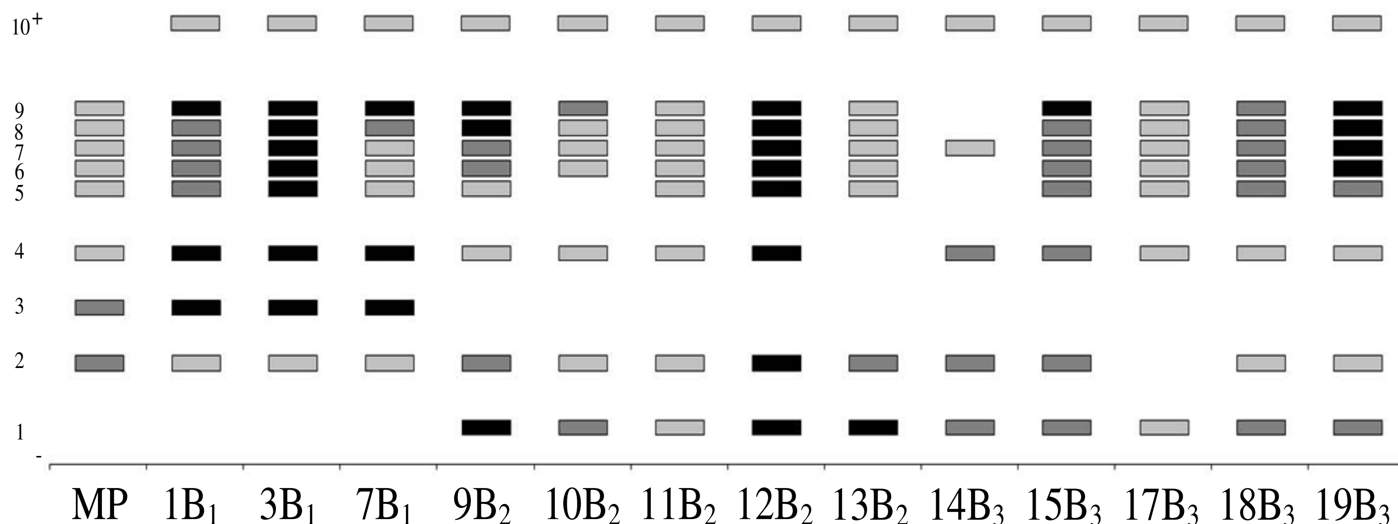

Figure 2. POD bands pattern for 13 somaclon plantlets of *E. microtheca* by B_1, B_2 and B_3 treatments. + and – are confine low and heavy molecules, respectively. MP is Mother Plant.

regeneration by B_5 treatment, POD alleles had similar behavior as in B_4 (Figure 4F).

SSR molecular marker

EMBRA06 primer yielded a very dense PCR product of over 150 bp with high repeatability on agarose gel (Figure 5). EMCRC09 primer resulted to a product below 500 bp (Figure 6). After loading EMBRA06 over denaturing polyacrylamide gel, only plantlet No. 23 yielded no band (Figure 7). The existence of polymorphism for plantlets 14 and 29, by EMCRC09 over denaturing polyacrylamide gel was clear, this band was about 280 bp (Figure 8).

DISCUSSION

NAA and Kin (1.0 mgl^{-1} each) resulted in direct regeneration induction and twigs explants regenerated without going through the callus formation stage. Adding 0.01 mgl^{-1} TDZ to B_2 treatment, increases the volume of calli in small amount and results in somatic embryogenesis. In other words, the effect of NAA and Kin in small amounts dominates TDZ. In B_3 treatment in which there is 0.1mgl^{-1} TDZ, somatic embryogenesis gets to its highest rate (52%). When the TDZ amount is 0.5 and 1 mgl^{-1}, calluses become compact and hard, regeneration decreases and there will be indirect regeneration induction. The results show that the types of *E. microtheca* regene-

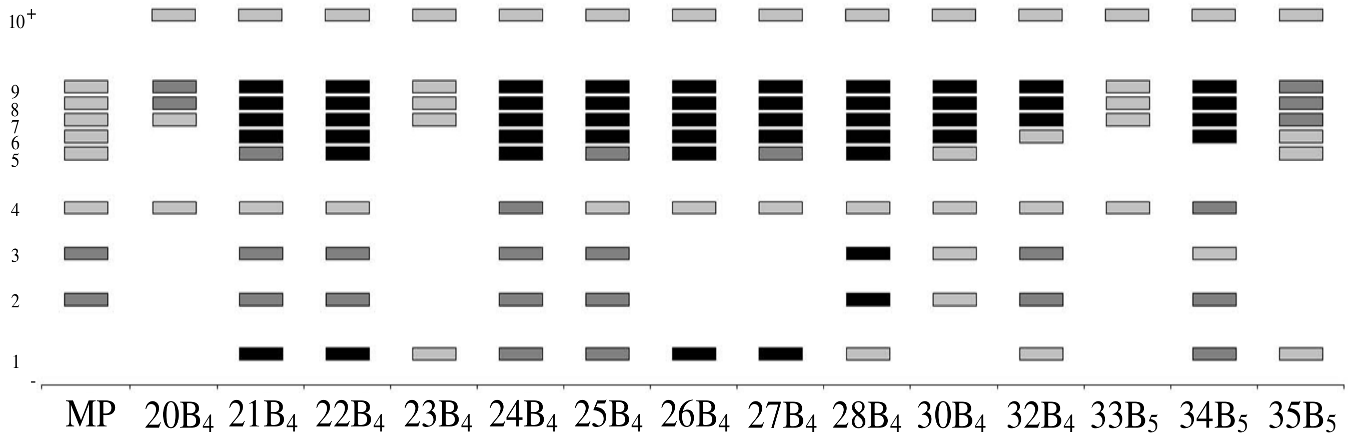

Figure 3. POD bands pattern for 14 somaclon plantlets of *E. microtheca* by B_4 and B_5 treatments. + and – are confine low and heavy molecules, respectively. MP is Mother Plant.

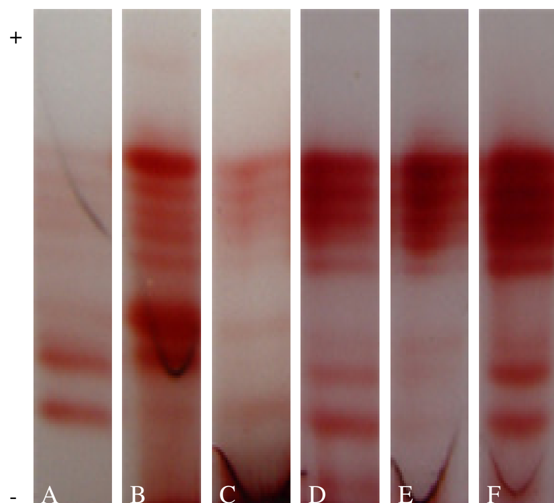

Figure 4. POD bands in somaclon plantlets of *E. microtheca*. A: Mother plant with two locus, dimer and tetramer. B: The alleles activity in B_1 treatment and seem of the new band on the confine low molecules. C: alleles activity in B_2 and B_3 treatments with deletion of the heterozygote allele from dimer locus and created other new band on the confine heavy molecules. D: Seeing again heterozygote allele in B_4 treatment. E: Decrease of the dimer locus activity in B_5 treatment. F: Intense activity at the both locus in B_4 and B_5 treatments. + and – are confine low and heavy molecules, respectively.

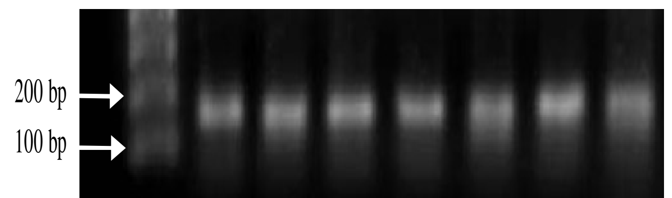

Figure 5. SSR bands for 7 somaclon plantlets of *E. microtheca* with EMBRA06 primer on the agarose gel (first sample is Size marker).

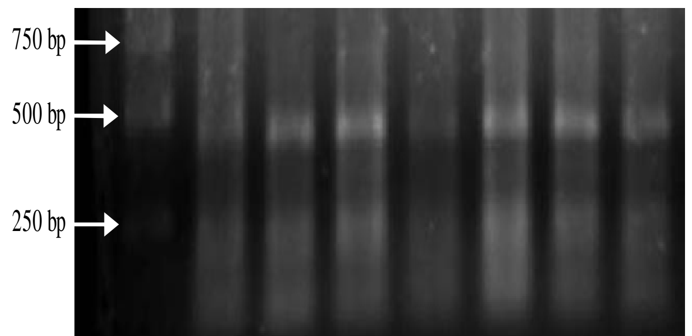

Figure 6. SSR bands for 7 somaclon plantlets of *E. microtheca* with EMCRC09 primer on the agarose gel (first sample is Size marker).

ration depend on TDZ. TDZ can highly induce synthesizing, gathering and modifying other produced cytokinins (Visser et al., 1992).

The rate of POD activity is reported to decease in direct regeneration of white pine cotyledon leaf explants using TDZ (Tang and Newton, 2005). This finding completely complies with the findings of this research, since the activity of alleles has been reduced and 3 alleles were invisible. Also entering the somatic embryogenesis phase, in which the relationship between embryo and calluses completely disappears, POD amount decreases remarkably. But there is not a clear difference between B4 and B5 in which there was indirect regeneration, so when the TDZ cytokinin increases, POD activity increases too. This finding supports the idea of TDZ adjustment

Figure 7. SSR bands with EMBRA06 primer on the 6% denaturing polyacrylamide gel for 33 somaclon plantlets of *E. microtheca* (M is Mother Plant).

Figure 8. SSR bands with EMCRC09 primer on the 6% denaturing polyacrylamide gel for 33 somaclon plantlets of *E. microtheca* (M is Mother Plant).

Table 4. Results of X^2 test.

X^2 test		EMBRA06		EMCRC09	
		Df	Probability	Df	Probability
POD bands	PEB2	1	$0.8446^{n.s}$	1	$0.8446^{n.s}$
	PTB5	1	$0.8446^{n.s}$	1	0.0001^{**}
	PTB4	1	$0.8446^{n.s}$	1	0.0001^{**}
	PTB3	-	-	-	-
	PTB2	1	0.0126^{*}	1	0.0126^{*}
	PTB1	1	$0.0778^{n.s}$	1	$0.0778^{n.s}$
	PDB3	1	0.0033^{**}	1	$0.7243^{n.s}$
	PDB2	1	$0.3778^{n.s}$	1	$0.3778^{n.s}$
	PDB1	1	$0.0778^{n.s}$	1	$0.5566^{n.s}$
	PEB1	1	$0.5566^{n.s}$	1	$0.5566^{n.s}$

P: POD, D: Dimer, T: Tetramer, E: Epigenetic, B: Band. *Significant in 5%; **significant in 1% and n.s non significant.

property for different types of regenerations (Visser et al., 1992). Our findings show that POD activity positively correlates with TDZ level. As the overall result, it seems that changes in POD activity, is an index for regeneration. A change in POD activity in somatic embryogenesis and organogenesis is considered as a marker (Preetha et al., 1995; Xiaoli et al., 1992). One epigenetic band (PEB2) will appear in all regenerations using NAA and Kin hormone. Meanwhile the plantlets influenced by TDZ hormone, create other epigenetic (PEB2) band. SSR profiles do not correlate with these two bands (Table 4) and the least Spearman rank coefficient exists between these two bands and molecular marker (Table 5). The highest coefficient for EMBRA06 primer with PDB2 allele from dimer locus is r = 0.56 which indicates the relative effect of proliferation sequences by this primer, over POD synthesis. But the EMCRC09 primer has significant affect on PTB4 and PTB5 alleles (Table 4), and there is a complete coefficient for them (Table 5). Thus, a close correlation exits between TDZ level and the presence of alleles from a tetramer locus.

In B_2 and B_3 treatments, activities of all alleles have been reduced and PDB2 allele has disappeared in all plantlets. So it can be said that 0.01 and 0.1 mg l^{-1} of TDZ have caused disappearance of a heterozygote allele from a dimer locus. This phenomenon could happen due to a repressor synthesis for POD promoter (Lagrimini and Klotz, 1997) or mutation (Larkin and Scowcroft, 1981). Low correlation between similarity coefficient matrixes of POD isoenzyme and microsatellites (r = 0.18) is very similar to the results of Sun et al. (1997), due to applying Mental test (1967). So Lagrimini and Klotz (1997) theory concerning phytohormone control of the POD promoter may be accurate.

Table 5. Spearman ranking coefficients.

Primer	EMBRA06	EMBRA06	EMBRA06	EMBRA06	EMBRA06	EMBRA06	EMBRA06	EMBRA06	EMBRA06	EMBRA06
POD bands	PEB1	PDB1	PDB2	PDB3	PTB1	PTB2	PTB3	PTB4	PTB5	PEB2
SRC	0	0.3333	0.1667	0.5556	0.3333	0.4714	-	0	0	0
Primer	**EMCRC09**	**EMCRC09**	**EMCRC09**	**EMCRC09**	**EMCRC09**	**EMCRC09**	**EMCRC09**	**EMCRC09**	**EMCRC09**	**EMCRC09**
POD bands	PEB1	PDB1	PDB2	PDB3	PTB1	PTB2	PTB3	PTB4	PTB5	PEB2
Primer	0	0	0.1667	0	0.3333	0.4714	-	1	1	0

(P: POD, D: Dimer, T: Tetramer, E: Epigenetic, B: Band, SRC: Spearman ranking coefficients).

The relationship between SSR sequences and POD synthesis can be estimated to be low in *E. microtheca*. However, microsatellites have more relationship with POD tetrameter locus compared to dimer locus. Also the alleles of tetrameter locus, under TDZ effect, have more stability and important role in POD synthesis.

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