© Triveni Enterprises, Lucknow (India)
ISSN : 0254-8704
http : //www.geocities.com/j_environ_biol/

J. Environ. Biol.
29(6), 841-844 (2008)
j_environ_biol@yahoo.com

Calli cultures from *Abies equi-trojani* (Aschers et Sinten) and changes in antioxidant defense system enzymes

Ercan Arican*, Gulruh Albayrak and Nermin Gozukirmizi

Department of Molecular Biology and Genetics, Faculty of Science, Istanbul University - 34134, Vezneciler-Istanbul, Turkey

(Received: April 11, 2007; Revised received: July 18,2007; Re-revised received: July 28, 2007; Accepted: August 10, 2007)

Abstract: *This study was conducted to explain difficulties of indirect regeneration of forest trees in tissue culture conditions. For this purpose, changes of antioxidant defense system enzymes; superoxide dismutase (SOD), peroxidase (POD) activities were determined during calli formation on young apical shoots of Abies equi-trojani (Aschers et Sinten). Young apical shoots were collected from naturally growing trees and cultured on two different media; Murashige and Skoog (MS) and McCown Woody plant medium (WPM) supplemented with growth regulators benzyl amino purine (BAP), 2,4-dichloro phenoxy acetic acid (2.4-D), kinetin (Kn) in various concentrations for callus induction. WPM media containing 1 mg ml^{-1} BAP and 1 mg ml^{-1} 2,4-D gave the best calli induction ratio (74 %) between tested combinations. POD and SOD enzyme activities were measured both on young shoot explants and 10 day-old calli derived from these explants. POD and SOD enzyme activities were higher, being 81.02% and 74.82%, respectively on calli when compared to shoots. The results showed that culture stress tolerated with increased antioxidant enzyme activities could be considered as protective physiological responses in calli cells.*

Key words: *Abies, Antioxidants, SOD activity, POD activity, In vitro*
PDF of full length paper is available with author ***(*earican@istanbul.edu.tr)***

Introduction

A number of factors affected plantlet regeneration, for instance seed variability, age of seedlings, and mode of application of growth substances. In terms of gymnosperm in contrast to angiosperm calli there is no developed standard tissue culture media. This problem is regarded as a major constraint in the full realization of the potentialities of biotechnology for forestry (Bomman, 1983). Vegetative propagation of *Abies* sp through cuttings is difficult due to rooting problems and the plagiotropic growth habit of the ramets (Nørgaard *et al*., 1993). Tissue culture methods are used in forestry for multiplying elite genotypes, although rooted cutting is still, operationally, the most effective propagation method available to many tree species (Ayan *et al*., 2006). In the genus *Abies,* the development of micropropagation has not yet resulted in practical applications, but plantlet regeneration was achieved in the hybrids *A. alba, A. balsamea, A. nordmaniana* and *A. fraseri,* and in the hybrids *A. alba x A. cephalonica* and *A. alba x A. numidica* (Guevin *et al*., 1994; Hristoforoglu *et al*., 1995; Guevin and Kirby, 1997; Aronen *et al*., 2000).

Abies species was found by Sintensis in 1883, and Boisier thought that it was a variety of *Abies alba*, then Ascherson and Sintensis classified it as a new species locating on Kazdaglari (Ida mountain), Turkey (Ucler *et al*., 2007). During plant cell, tissue and organ culture one of the well defined problems is the recalcitrance (absence of organogenic potential); the terms used to describe this phenomenon are known to be genotype and environment dependent. However, changes in DNA caused by oxidative stress which can lead to recalcitrance, loss of competence, hyperhydricity and somaclonal variation has been proposed to explain this phenomenon recently (Cassels and Curry, 2000). To counteract the damaging effects of stress situations plants have developed specific molecular and biochemical mechanisms, the investigation of which has been a major research topic for several years (Blumwald, 2000; Seki *et al.,* 2003; Zhu, 2003). Stress-resulting from the generation of reactive oxygen species, such as superoxide (O^{-}_{2}), peroxide hydrogen (H_2O_2) and hydroxyl radicals (OH^{-}) is detrimental to plant survival under stress environment. To counteract the toxicity of ROS, a highly efficient antioxidative defense system, including both nonenzymic (*e.g.* ascorbate, carotenoides, α-tocopherol) and enzymic constituents [SOD (EC 1.15.1.1), POD (EC 1.11.1.7), CAT (EC 1.11.1.6)], is present in plant cells and plays an important role in defending plants from activated oxygen species damage (Ming *et al.,* 2003). *In vitro,* various media factors have been shown to induce stress, including hormones and mineral nutrients (Ziv, 2003).

In this study, we reported accumulation of SOD and POD enzymes during calli formation from shoot cuttings of *A. equi-trojani* as possible stress factors for recalcitrance of calli material.

Materials and Methods

Plant material: Young *A. equi-trojani* shoot samples were collected from top of the trees (~3-5 m) (Kazdaglari-Ceyizderesi/Ida Mountain) (N 39°42.878', E 026°51.926', 1450 m) Natural Park/Edremit-Balikesir,Turkey.

Surface sterilization and culture conditions: The young shoots were first rinsed with 70% ethanol for 15 min, then surface sterilized in 50% sodium hypochloride containing 0.2% Tween 20 for 45 min. After sterilization the explants were rinsed five times with sterile distilled water. These explants were then cultured on

Table - 1: Media tested for the induction of calli from apical shoot explants of *A. equi-trojani*

Medium	Growth regulators (mg l^{-1})		
	BAP	2,4-D	Kn
MS1	1	1	
MS2	1	3	
MS3	1		1
MS4	1		3
MS5		1	0.1
MS6		4	0.1
MS7		2	0.5
MS8		2	2
WPM1	1	1	
WPM2	1	3	
WPM3	1		1
WPM4	1		3
WPM5		1	0.1
WPM6		4	0.1
WPM7		2	0.5
WPM8		2	2

Kn = Kinetin

Table - 2: Calli induction ratios of *Abies equi-trojani* young shoots on MS and WPM media

Medium	Growth regulators (mg l^{-1})			No. of explants	No. of callus	Frequency (%) of forming callus
	BAP	2,4-D	Kn			
MS1	1	1	–	50	21	42 ± 0.82
MS2	1	3	–	50	2	4 ± 1.41
MS3	1	–	1	50	3	6 ± 0.82
MS4	1	–	3	50	4	8 ± 1.41
MS5	–	1	0.1	50	8	16 ± 0.82
MS6	–	4	0.1	50	1	2 ± 0.82
MS7	–	2	0.5	50	2	4 ± 0.00
MS8	–	2	2	50	4	8 ± 0.82
WPM1	1	1	–	50	37	74 ± 2.16
WPM2	1	3	–	50	11	22 ± 1.41
WPM3	1	–	1	50	11	22 ± 3.74
WPM4	1	–	3	50	11	22 ± 1.63
WPM5	–	1	0.1	50	26	52 ± 3.27
WPM6	–	4	0.1	50	6	12 ± 2.16
WPM7	–	2	0.5	50	9	18 ± 0.82
WPM8	–	2	2	50	9	18 ± 0.82

Table - 3: SOD and POD activities on 0 day young shoots and 10 day old calli of *A. equi-trojani*

Parameters	Explants	Values
SOD	0 day young shoots	10.88 ± 2.99^{1}
(Unit mg protein $^{-1}$)	10 day old calli	19.02 ± 3.02^{2}
POD	0 day young shoots	12.33 ± 1.25^{2}
(Unit mg protein $^{-1}$)	10 day old calli	22.32 ± 1.12^{1}

Experiments were set up in a randomized design. Each treatment consisted of 30 embryos and each experiment was carried out three times. The data shown represented the mean ± SE of three independent ANOVA was performed on the results of each experiment and the data were analyzed using Duncan's multiple range test ($^{1}p< 0.01$, $^{2}p<0.001$)

MS (Murashige and Skoog, 1962) and WPM (Lloyd and McCown, 1981) supplemented with different concentration and combinations of BAP, 2,4-D, Kinetin either alone or in combination (Table 1). The basal medium for all experiments consisted of MS and WPM mineral nutrients and MS vitamin mixture with 30 gl^{-1} sucrose and 3% agar. pH of the mediums was adjusted to 5.7 prior to autoclaving at 121°C for 20 min. Explants were incubated on sterile media in Petri dishes (100 x 15 mm) containing 25 ml of medium sealed with parafilm and maintained at 25°C in a growth chamber with 30 $\mu molm^{-2}s^{-1}$ illumination from cool-white fluorescent lambs for 16 hr photoperiod.

Measurement of enzyme activities: Plant samples (shoots and calli, 1 g) were homogenized in 1 ml of 50 mM sodium phosphate (pH 7.0) that contained 1% PVP-40. The homogenate was centrifuged at 15,000 g for 30 minutes. The supernatant of the both plant samples were collected and stored at -80°C for further analyses.

POD activity was based on the determination of guaiacol oxidation (extinction coefficient 26.6 mmol l^{-1} cm^{-1}) at 470 nm by H_2O_2. The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 16 mM guaiacol and 10 ml 10% H_2O_2 in a 3 ml volume. The reaction was initiated by adding plant extract and was followed for 2 min (Fridovich, 1986).

SOD activity assay was based on the method of Beauchamp and Fridovich (1971), which measures the inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm wave length. One unit of enzyme activity was defined as the quantity of SOD required to produce a 50% inhibition of reduction of NBT, and the specific enzyme activity was expressed as unit's mg^{-1} protein. The reaction mixture contained 50 mM Na phosphate buffer (pH 7.8), 33 mM NBT, 10 mM L-methionine, 0.66 mM EDTA and 0.0033 μM riboflavin. Reactions were carried out at 25°C under light intensity which was about 300 $\mu mol^{-1}m^{-1}s^{-1}$ through 10 min. Electrophoreses were performed using native acrylamide gels and SOD activity was showed by immersion of the gel in riboflavin and N, N, N', N'-teramthylethylenediamine, exposure to light to produce superoxide radicals and staining with nitroblue tetrazolium, as described by Fridovich (1986).

Statistical analysis: The data were analyzed statistically using ANOVA. Dunnet's test (between control and treatment groups) and Student Newman-Keuls test (between treatment groups) were used for multiple comparisons. The data analyzed were those from a minimum of three in dependent experiments. For the statistical evaluation of the results, the significance was accepted at the probability level of $p<0.01$ (Shun *et al.,* 2003).

Results and Discussion

In this study; the calli formation and changes in antioxidant defense system enzymes; SOD, POD activities were studied in *Abies equi-trojani* (Aschers et Sinten) young apical shoots. WPM media containing 1 mg l^{-1} BAP and 1 mg l^{-1} 2,4-D gave the best calli induction ratio between tested combinations (Table 2). POD and

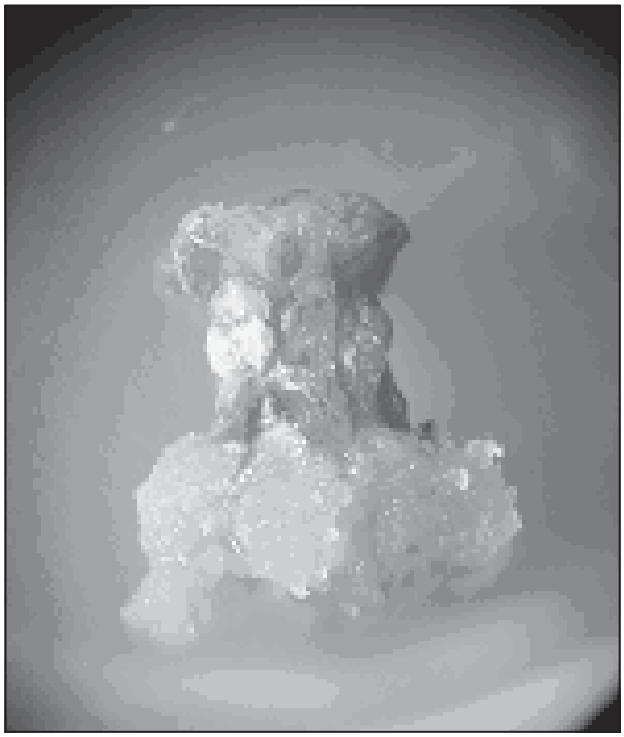

Fig. 1: Callus formation from *A. equi-trojani* young shoot explants after 10 days on WPM medium

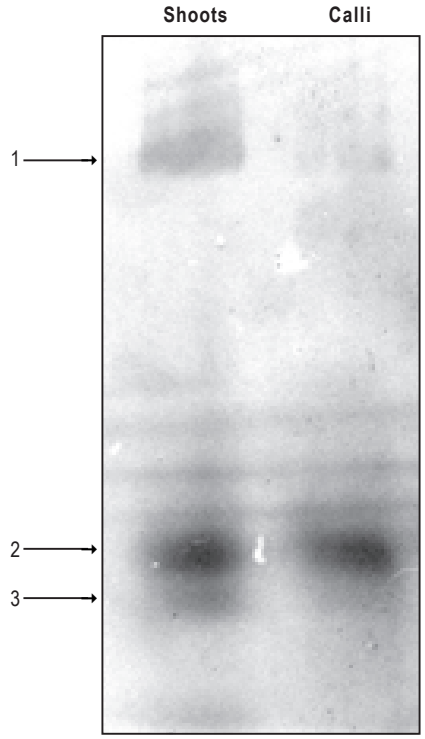

Fig. 2: SOD isozymes (1,2,3) activity patterns on native polyacrylamide

SOD enzyme activities were measured both on young shoot explants and 10 day-old calli derived from these explants. Enzyme activities were higher on calli when compared to shoots (Table 3). The results showed that culture stress tolerated with increased antioxidant enzymes activities could be considered as protective physiological responses in calli cells.

Of two different media in eight various combinations used for calli inductions from young apical shoots of *Abies equi-trojani*, WPM1 medium supplemented with 1 mg l^{-1} BAP and 1 mg l^{-1} 2,4-D gave the best calli induction ratio (74%). This calli induction ratio was higher (76.2%) than MS medium with the same concentration (Table 2).

Calli inductions started from the ends of shoot explants of *A. equi-trojani* (Fig. 1). They became brownish and died on 15 days after callus induction from explants in all tested conditions. Soluble protein isolation was realized from shoots and 10 day-old calli. POD activity was determined via a spectrophotometer. POD activity was higher in calli than in shoots (81.02%) (Table 3), SOD activity increased (74.82%) significantly in the calli after 10 days. In addition, native gel electrophoresis was performed to confirm increase in SOD activity. Each experiment was repeated three times and one accompanying picture of these experiments was presented (Fig. 2). First and third isozyme bands were more compact in calli than in the young shoots. Several reactive oxygen species (ROS) are continuously produced in plants as by products of aerobic metabolism. Depending on the nature of the ROS species, some are highly toxic and rapidly detoxified by various cellular enzymatic and nonenzymatic mechanisms (Apei and Hirt, 2004). Active oxygen species (AOS) are also involved in various aspects of seed physiology. Their generation, which occurs during seed desiccation, germination and aging, may lead to oxidative stress and cellular damage, resulting in seed deterioration. However, cells are endowed with detoxifying enzymes and antioxidant compounds that scavenge AOS and participate in seed survival. The detoxifying mechanisms play a key role in acquisition of desiccation tolerance of developing seeds, completion of seed germination and seed storability (Bailey, 2004). Similarly, increase in SOD activity in calli development could be seen as a result of antioxidant defense system of plants.

Cheng and Song (2006), reported that there were considerable differences in SOD activity among the leaves of maize, mung bean and wheat, as well as between different organs of the same species. These results demonstrate that the SOD activity varies with species and organs. In our study; the SOD enzyme patterns were different in calli from shoots on polyacrylamide gel (Fig. 2). This may also be explained by the fact plants have multiple genes encoding SOD and the genes expressing SOD can be induced by some stress conditions, such as high light, low temperature, increased O_2 concentration and xenobiotic and fungal attack.

Plant cells can detect a physical disturbance at their cell surfaces and initiate the oxidative burst reaction in response (Yahraus *et al*., 1995). During the habituation in plant cell culture adaptation occurs to free radical attacks; such changes could explain, at least in part, the range of variability found in plant cells, tissue and organs in culture, namely, recalcitrance including loss of cell competence (Hagege, 1997). The principal defenses against these reactive

molecules and free radicals in plants include detoxifying enzymes (catalase, superoxide dismutase, *etc.*) and also lower molecular weight secondary products with antioxidant activity (Larson, 1995). In addition to enzymes mentioned above, some molecules such as ascorbate is an antioxidant and, in association with other components of the antioxidant system, protects plants against oxidative damage (Smirnoff, 1996). The problem with indirect regeneration from forest trees in tissue culture conditions could be the result of increase in antioxidant enzyme activities as an injury response on explants. For preventing increment of these enzyme activites; many antioxidant chemicals (ascorbic acid and active charcoal) can be added into media. Efficient regeneration is the most important step in the biotechnological improvement of forest trees to overcome the difficulties in regeneration, research on antioxidant enzyme systems could be beneficial.

Acknowledgments

The present work was supported by the Research Fund of Istanbul University. Project No. 24/27082002 and UDP- 740/ 11052006.

References

Apei, K. and H. Hirt: Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.,* **55**, 373-399 (2004).

Aronen, T.S., J. Krajnakova, H.M. Haggman and L.A. Ryynanen: Genetic fidelity crypreserved embryogenic cultures of open-pollinated *Abies cephalonica. Plant Sci.,* **142**, 163-172 (2000).

Ayan, S., A. Sivacioglu and N. Bilir: Growth variations of *Paulownia* Sieb. and Zucc. species and origins at the nursey stage in Kastamonu-Turkey. *J. Environ. Biol.,* **27**, 499-504 (2006).

Bailey, C.: Active oxygen species and antioxidants in seed biology. *Seed Sci. Res.,* **14**, 93-107 (2004).

Beauchamps, C. and I. Fridovich: Superoxide dismutase: Improved assays and applicable to acrylamide gels. *Anal. Biochem.,* **3**, 248-254 (1971).

Blumwald, E.: Sodium transport and salt tolerance in plants. *Curr. Opin. Cell Biol.,* **12**, 431-434 (2000).

Bomman, C.H.: Possibilities and constraints in the regeneration of trees from cotyledonary needles of *Picea abies in vitro*. *Physiol. Plant,* **57**, 5-16 (1983).

Cassells, A.C. and R.F. Curry: Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: Implications for micropropagators and genetic engineers. *Plant Cell Tissue Organ Culture*, **64**, 145-157 (2000).

Cheng, H.Y. and S.Q. Song: Species and organ diversity in the effects of hydrogen peroxide on superoxide dismutase activity *in vitro*. *J. Integrative Plant Biol*., **48**, 672-678 (2006).

Fridovich, I.: Biological effects of the superoxide radical. *Arch. Biochem. Biophysics*, **247**, 1-11 (1986).

Guevin, T.G. and E.G. Kirby: Induction of embryogenesis in cultured mature zygotic embryos of *Abies fraseri* (Pursh) Poir. *Plant Cell Tiss. Org. Cult.,* **49**, 219-222 (1997).

Guevin, T.G., V. Micah and E. Kirby: Somatic embryogenesis in cultured mature zygotic embryos of *Abies balsamea. Plant Cell Tiss. Org. Cult.,* **37**, 205-208 (1994).

Hagege, D.: Habituation in plant cell cultures: Adaptation to free radical attacks. *CR Soc. Biol.,* **189**, 1183-1190 (1997).

Hristoforoglu, K., H. Schmidt, B. Nordenkampf: Development and germination of *Abies alba* somatic embryos. *Plant Cell Tiss. Org. Cult.,* **40**, 277-284 (1995).

Larson, R.A.: Plant defences against oxidative stress. *Arch. Insect. Biochem. Physiol*., **29**, 175-186 (1995).

Lloyd, G. and B. McCown: Commercially feasible micropropagation of Mountain laurel, *Kalmia latifolia* by use of shoot tip culture. *Proc. Plant Prop. Soc.,* **30**, 421-427 (1981).

Ming, L., W. Gen Xuan and L. Jiou Sheng: Application of external calcium in improving the PEG-induced water stress tolerance in liquorice cells. *Bot. Bull. Acad. Sci.,* **44**, 275-284 (2003).

Murashige, T. and F. Skoog: A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant,* **15**, 473-497 (1962)

Norgaard, J.V., S. Baldursson and P. Krogsrupt: Genotypic differences in the ability of embryogenic *Abies nordmanniana* cultures to survive cryopreservation. *Silvea Genet.,* **42**, 93-97 (1993).

Seki, M., A. Kamei, K.Yamaguchi-Shinozai and K. Shinozaki: Molecular responses to drought, salinity and frost: Common and different plants for plant protection. *Curr. Opin. Plant Biotechnol.,* **14**, 194-199 (2003).

Shun, Z., A. Silverberg, K. Chang and P. Ouyang: Dunnett's many-to-one test and least square means. *J. Biopharm. Stat.,* **13**, 17-28 (2003).

Smirnoff, N.: The function and metabolism of ascorbic acid in plants. *Ann. Bot*., **78**, 661-669 (1996).

Ucler, A.O., Z. Yucesan, A. Demirci, H. Yavuz and E. Oktan: Natural tree collectives of pure oriental spruce [*Picea orientalis* (L.) Link] on mountain forests in Turkey. *J. Environ. Biol.,* **28**, 295-302 (2007).

Yahraus, T., S. Chandra, L. Legendre and P.S. Low: Evidence for a mechanically induced oxidative burst. *Plant Physiol.,* **109**, 1259-1266 (1995).

Zhu, J.K.: Regulation of ion homeostasis under salt stress. *Curr. Opin. Plant Biol.,* **6**, 441-445 (2003).

Ziv, M.: Vitrification. *In*: Morphological and physiological disorders of *in vitro* plants (*Eds.:* P.C. Debergh and K.H. Zimmermann). Kluwer Acad. Publishers. pp. 45-70 (2003).