BIOLOGIA PLANTARUM 56 (2): 357-361, 2012

BRIEF COMMUNICATION

Changes in antioxidative enzymes activities during *Tacitus bellus* direct shoot organogenesis

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Abstract

Changes in antioxidative enzymes activities during *Tacitus bellus* direct shoot organogenesis from leaf explants were examined. During the early stages of shoot organogenesis there was a decrease in superoxide dismutase (SOD) and an increase in catalase (CAT) activity, and later during organogenesis there was an increase in peroxidase (POD) and polyphenol oxidase (PPO) activity. Two highly regulated turning points may be distinguished regarding activities and isoforms of antioxidative enzymes: the initiation of shoot organogenesis and the shoot bud formation. Our data suggest the role of specific CAT, POD, SOD and PPO isoforms in separate processes during *T. bellus* direct shoot organogenesis.

Additional key words: catalase, peroxidase, polyphenol oxidase, superoxide dismutase.

Organogenesis is a complex process accompanied with expression of various genes and protein synthesis. Organ initiation and development in plant explants involves promotion of meristematic activity, its maintenance in some regions and its concomitant suppression in regions of maturation (Kay and Basile 1987). Reactive oxygen species (ROS) and their scavenging enzymes participate in protection against pathogens or abiotic stress (Hendry and Crawford 1994), in regulation of plant growth and developmental processes (Mitrović and Bogdanović 2008), and in processes linked with development, differentiation and growth of plant cells during morphogenesis *in vitro*. It was shown that H_2O_2 content and activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) are correlated with bud primordium formation in strawberry callus (Tian *et al*. 2003). Involvement of CAT and POD in direct adventitious shoot formation was detected in eastern white pine (Tang and Newton 2005), in *Gladiolus hybridus* (Gupta and Datta 2003/4). Kay and Basile (1987) examined the role of specific isoperoxidases in different developmental processes during organogenesis in tobacco. The role of polyphenol oxidase (PPO) in defence against pathogens, in cell division and root primordia development was suggested, but its physiological function is not clear (Mayer 2006). *In vitro* culture is a useful model system for the investigations of morphological, biochemical and molecular processes linked with early development of plants. In this study we used *Tacitus bellus* as a model plant. Earlier we reported *T. bellus in vitro* micropropagation and *ex vitro* acclimation (Mitrović *et al*. 2005). The aim of this study was to examine the correlation between activities of antioxidative enzymes and different phases of *T. bellus*

Received 27 April 2010, *accepted* 22 October 2010.
Abbreviations: BAP - benzylaminopurine; CAT - catalase; EDTA - ethylenediaminetetraacetic acid; NAA - naphtaleneacetic acid; POD - peroxidase; PPO - polyphenol oxidase; PVP - polyvinylpyrrolidone; SOD - superoxide dismutase; SDS - sodium dodecyl sulfate; TRIS - tris(hydroxymethyl)aminomethane.
Acknowledgements: This work was supported by a grant (No. 173017 and 173015) from the Ministry of Education and Science of the Republic of Serbia.
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shoot organogenesis *in vitro,* in order to elucidate the involvement of active oxygen species in shoot organogenesis.

The experiments were carried out on *Tacitus bellus* (L.) Moran and J. Meyrán, syn. *Graptopetalum bellum*, fam. *Crassulaceae*. Primary explants were entire lower rosette leaves from *in vitro* multiplied and on hormone free medium rooted plants (Mitrović *et al.* 2005). They were placed abaxialy, on MS (Murashige and Skoog 1962) medium supplemented with sucrose (30 g dm^{-3}), benzylaminopurine (BAP) (0.1 mg dm^{-3}) and naphtalene-acetic acid (NAA) (0.1 mg dm^{-3}), gelled with agar (0.7 %) and cultured during 60 d. Irradiance was 70 μmol m^{-2} s^{-1}, temperature was 25 ± 2 ºC. The experiment was repeated twice.

For histological analysis, explants were fixed in formalin + acetic acid + ethanol (10:5:85), dehydrated in graded ethanol series and embedded in paraffin wax at 58 °C. Sections (8 μm thick) were stained with haematoxylin and photographed under *Leitz DMRB* photomicroscope (*Leica*, Wetzlar, Germany).

For enzyme analysis 4 samples (5 explants per sample) were collected per time point (0, 10, 20, 40 and 60 d of culturing) and powdered in the liquid nitrogen in a mortar. The powder was resuspended in the extraction buffer (0.1 M TRIS-HCl, pH 7.6, containing 1 mM dithiotreitol, 1 mM EDTA, 0.5 % *Tween 80* and 2 % PVP, in 1:5 (m/v) ratios). The homogenates were stirred on ice for 30 min and centrifuged at 12 000 *g* for 10 min. The supernatant was desalted on *Sephadex G-25* (*NAP-5* column, *Amersham Biosciences*, Steinheim, Germany) and used for enzyme activity measurements and zymogram detection. Enzyme activities were determined spectrophotometrically using a *Shimadzu* (Tokyo, Japan) UV-160 spectrophotometer. Peroxidase activity was determined with guaiacol as a substrate (Chance and Maehly 1955). The assay mixture contained 50 mM acetate buffer (pH 4.5), 92 mM guaiacol, 18 mM H_2O_2 and 0.01 cm^3 enzyme extract. The turnover of guaiacol was monitored at 470 nm and reaction rate calculated from the coefficient of absorbance for guaiacol of 25.5 mM^{-1} cm^{-1}. Catechol oxidase activity was determined using 40 mM catechol in 100 mM phosphate buffer (pH 6.5), and 0.1 cm^3 enzyme extract, at 410 nm. One unit of catechol oxidase activity was defined as the change in absorbance of 0.001 min^{-1}. Superoxide dismutase activity was determined by the ferricytochrome *c* method using xantine/xantine oxidase as the source of superoxide radicals, in the 100 mM K-phosphate buffer (pH 7.8) (McCord and Fridovich 1969), in the presence of 100 μM EDTA and 20 μM sodium azide. The presence of CuZn-, Fe- and Mn-SODs was investigated using KCN and H_2O_2 at 5 mM final concentrations (Asada *et al*. 1975). The activity of CAT was determined by the rate of H_2O_2 disappearance at 240 nm (Claiborne 1984). Coefficient of absorbance for H_2O_2 was 4.32 μM^{-1} cm^{-1}. One unit of CAT activity is defined as the amount of enzyme that decomposes 1 μmol of H_2O_2 per min at 25 ºC and pH 7.0. The enzyme activities were referred to fresh mass of the samples, and to the protein content determined by the method of Lowry *et al*. (1951) with bovine serum albumin as the standard.

Peroxidase, SOD and catechol oxidase isoenzymes were separated in a pH gradient from 3 to 9 (using 5 % ampholite solution) on a 7.5 % polyacrylamide gel. Peroxidase isoenzymes were stained on gel with 0.6 mM 4-chloro-1-naphthol and 5 mM H_2O_2 in Na-acetate buffer (pH 5.5) for 10 min at 25 °C. SOD isoenzymes were stained according to Beauchamp and Fridowich (1971), by 30 min incubation of gels with $2.5 \cdot 10^{-5}$ M NBT and $8.44 \cdot 10^{-6}$ M riboflavin in dark, and subsequent irradiation with UV light. In order to distinguish between Mn-, Cu/Zn- and Fe-SOD isoforms, activity staining was performed in gels previously incubated at 25°C for 40 min in 100 mM K-phosphate buffer (pH 7.8) containing 5 mM KCN or 5 mM H_2O_2. The catechol oxidase isoenzymes were stained with 5 mM L-DOPA in 0.1 M phosphate buffer (pH 6.5) for 1 h at 25 °C (Gillespie *et al*. 1991). Polyacrylamide gel electrophoresis was carried out under non/denaturing conditions in gels containing 8 % polyacrylamide with a 4 % stacking gel (Laemmli 1970) except that SDS was excluded. Equal volume of all samples were loaded on the gels. The CAT isoenzymes on gel were detected by the method of Woodbury *et al*. (1971).

Several succulent plants have been successfully propagated *in vitro* by direct or indirect shoot multiplication (Malda *et al*. 1999), although most of these species are often difficult to grow or propagate. Earlier we reported *T. bellus* micropropagation using different media for shoot induction and shoot multiplication (Mitrović *et al*. 2005). In this study separate morphogenic events related to direct *T. bellus* shoot organogenesis from leaf explants were obtained on the same medium - MS with BAP (0.1 mg dm^{-3}) and NAA (0.1 mg dm^{-3}), during 60 d. Histological examination revealed four main morphogenic stages of *T. bellus* shoot organogenesis: initiation of shoot organogenesis as early as after 10 d, meristemoids formation after 20 to 30 d, shoot buds formation after 40 d and rosettes formation after 60 d of culturing (Fig. 1*A-D*). From culture establishment until *de novo* rosette formation fresh mass of explants increased 22-fold, compared to just 5-fold increase in dry mass (Table 1). Protein content was low in primary explants. It increased markedly during organogenesis (Table 1). In primary explants we registered no CAT or POD activity, low PPO activity, while SOD activity was very high (Table 1). With the start of culturing on BAP and NAA switch in antioxidative enzymes activities is visible. Two highly regulated turning points may be distinguished regarding activities and isoformes of antioxidative enzymes, suggesting precise regulation of separate processes during *T. bellus* shoot organogenesis. First turning point is the initiation of shoot organogenesis

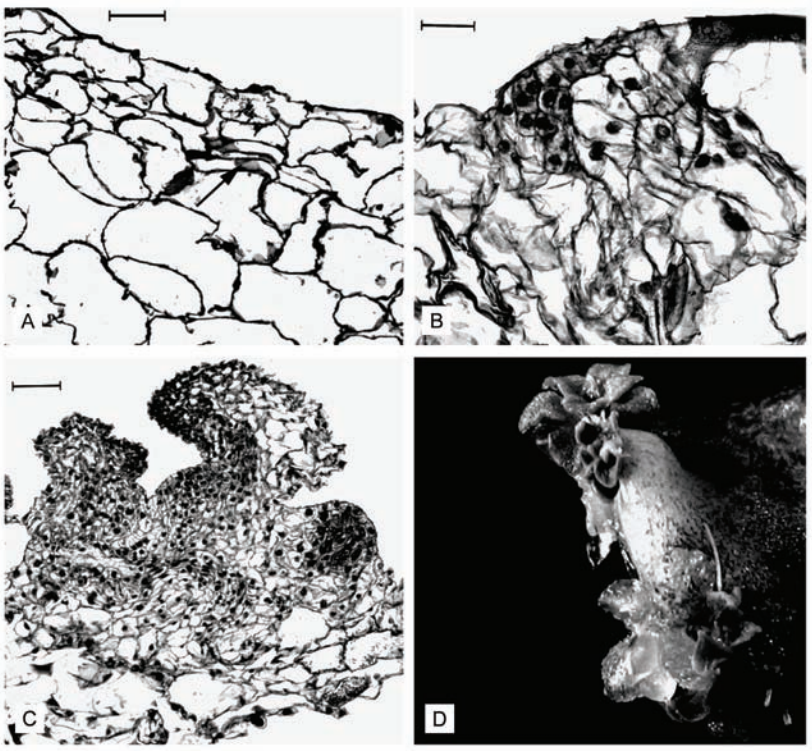

Fig. 1. Leaf explant of *T. bellus* placed on MS medium supplemented with 0.1 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA. *A* - Cross section of explant at 10th day of culture; the periclinal cell divisions in sub-epidermal cell layers is marked by an *arrow* (*bar* = 80 μm). *B* - Meristemoid on the surface of explant at 20th day of culture, the underlying cells are enlarged, highly vacuolated and poor in content (*bar* = 40 μm). *C* - Shoot buds at different stages of development after 40 d of culture (*bar* = 80 μm). *D* - Well formed rosettes on the surface of the explant after 60 d of culture.

Table 1. Changes in fresh and dry mass, protein content, catalase, peroxidase, superoxide dismutase and polyphenol oxidase activities and isoforms during *T. bellus* shoot organogenesis. All parameters were measured in primary explants and after 10, 20, 40 and 60 d of culturing on MS with BAP (0.1 mg dm^{-3}) and NAA (0.1 mg dm^{-3}). Individual isoforms are characterized by pI values. Means ± SE (n = 4); * - values significantly different from primary explants (0 d), using *ANOVA* test at 0.05 level of significance.

Parameters	Units	0 d	10 d	20 d	40 d	60 d
Fresh mass	[g]	0.22 ± 0.01	0.34 ± 0.025	0.37 ± 0.02	2.83 ± 0.29*	4.87 ± 0.34*
Dry mass	[g]	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00*	0.07 ± 0.02*	0.08 ± 0.01*
Protein	[g g^{-1}(f.m.)]	0.19 ± 0.05	2.77 ± 0.13*	2.78 ± 0.14*	0.28 ± 0.16*	1.61 ± 0.20*
CAT	[U g^{-1}(f.m.)]	-	61.10 ± 4.08*	49.95 ± 6.62*	61.72 ± 3.68*	37.16 ± 5.87*
	[U mg^{-1}(prot.)]	-	28.71 ± 3.40*	20.43 ± .76*	26.11 ± 2.63*	25.21 ± 0.81*
POD	[U g^{-1}(f.m.)]	-	1.32 ± 011	0.80 ± 0.03	28.66 ± 4.26*	22.16 ± 4.76*
	[U mg^{-1}(prot.)]	-	0.60 ± 0.15	0.33 ± 0.05	25.53 ± 9.05*	16.02 ± 4.93*
	pI	4.5	4.2, 4.5, 9.0	4.2, 4.5, 9.0	8.0, 8,5, 9.0	8.0, 8,5, 9.0
SOD (total)	[U g^{-1}(f.m.)]	1011.9 ± 28.2	378.5 ± 28.6*	426.8 ± 2.7*	76.77 ± 4.27*	43.53 ± 8.94*
	[U mg^{-1}(prot.)]	6285.0 ± 486.2	167.9 ± 35.2*	175.3 ± 21.7*	32.15 ± 5.77*	37.15 ± 10.1*
Mn-SOD	[U g^{-1}(f.m.)]	732.4 ± 18.6	335.8 ± 23.5*	400.4 ± 3.0*	65.10 ± 3.49*	42.66 ± 8.02*
	[U mg^{-1}(prot.)]	4701.9 ± 1378.9	151.9 ± 36.9*	164.3 ± 20.2*	29.91 ± 3.68*	30.80 ± 9.02*
	pI	3.7-8.6, 28	3.7-8.6, 28	3.7-8.6, 28	3.7-8.6, 28	3.7-8.6, 28
CuZn-SOD	[U g^{-1}(f.m.)]	297.4 ± 39.0	23.86 ± 13.10*	26.48 ± 3.06*	10.11 ± 1.48*	8.03 ± 2.44*
	[U mg^{-1}(prot.)]	1583.1 ±147.5	20.45 ± 6.79*	10.95 ± 1.90*	9.28 ± 5.03*	6.35 ± 1.74*
	pI	3.7, 3.9, 8.0	3.7, 3.9, 8.0	3.7, 3.9, 8.0	3.7, 3.9, 8.0	3.9, 8.0
Fe-SOD	pI	4.3	4.3	4.3	4.3	4.3
PPO	[U g^{-1}(f.m.)]	64.90 ± 5.46	131.5 ± 15.40*	86.58 ± 2.46*	313.9 ± 23.88*	373.0 ± 22.3*
	pI	9.0	9.0	9.0	8.5, 9.0	8.5, 9.0

(10^{th} day) and second is shoot buds formation (40^{th} day). At the time when the initiation of shoot organogenesis occurred, CAT activity increased (Table 1) and two CAT isoforms appeared (data not shown). Further, one acidic and one basic POD isoform appeared. SOD activity decreased to 1/3 of its value in primary explants coinciding with significant decrease in intensity of most SOD isoform bands and an increase in PPO activity (Table 1). During shoot buds formation, small increase in CAT activity occurred. The POD activity was maximum and two acidic POD isoforms disappeared and two new basic isoforms appeared (Table 1). At the same time, some SOD isoforms disappeared and PPO activity increased significantly by the appearance of new basic isoform (Table 1).

The capacity for ROS to serve as signals adds to the importance of antioxidants to specifically regulate different ROS in various cellular locations (Dat *et al*. 2000). There are several antioxidant enzymes in plants located in different plant cell compartments. Catalase has a high reaction rate but a low affinity for H_2O_2, thereby only removes the bulk of H_2O_2. In contrast peroxidases has a higher affinity for H_2O_2, allowing for the scavenging of small amounts of H_2O_2 in more specific locations (Dat *et al*. 2000). High CAT and POD activity could be correlated with differential processes during shoot induction (Gaspar 1995). We registered the decrease in SOD activity during *T. bellus* shoot organogenesis while increase in CAT (day 10) and POD (day 40) activities (Table 1). Similarly, in gladiolus (Gupta and Datta 2003/4), *Albizia odoratissima* (Rajeswari and Paliwal 2008), strawberry (Yonghua *et al*. 2005) and *Crocus sativus* (Vatankhah *et al.* 2010) increase in CAT and POD activity and concomitant decrease in SOD activity during shoot organogenesis were noticed. These data suggest that low H_2O_2 content might be involved in regulation of shoot organogenesis, which was confirmed by the results obtained by exogenous application of H_2O_2 in gladiolus (Gupta and Datta 2003/4). Decline in H_2O_2 accumulation at the time of shoot bud initiation in strawberry callus reported by Tian *et al*. (2003), was accompanied with constant decrease in superoxide content and with increase in POD but decrease in CAT and SOD activities. Similarly, later stages of *Pinus strobus* shoot bud formation were accompanied with increase in POD and a decrease in CAT activities (Tang and Newton 2005). The absence of POD and low PPO activities in *T. bellus* primary explants and their increase not until shoot bud formation (Table 1) argue in favour of Andersone and Ievinsh (2002) and Sujatha *et al*. (2000) findings. Low polyphenol oxidase and peroxidase activities coincide with increased morphogenic potential in mature *Pinus silvestris* buds (Andersone and Ievinsh 2002), while peak activities of peroxidase and polyphenol oxidase are associated with formation of meristematic centres during shoot regeneration in *Jatropa integerrima* (Sujatha *et al*. 2000). Changes in CAT activity during shoot organogenesis are reported for many species, but none for correlation of some CAT isoforms with separate phases during shoot organogenesis. Two CAT isoforms present in all samples from 10 to 60 d, and missing in samples of primary explants suggest their participation in all differentiation processes during *T. bellus* shoot organogenesis. PODs have a very important role in physiological processes in plants (Gaspar *et al*. 1991), but their exact relationship to developmental events is often obscure by their extensive polymorphism in a single plants species. It is therefore very important to select PODs associated with plant development (Jackson and Ricardo 1998). PODs are involved in auxin and ethylene metabolism and cell wall reconstruction, therefore considered as possible marker of morphogenesis. In our study histological examination enabled us to link specific POD isoforms with separate processes during organogenesis. The appearance of new POD acidic isoform (pI 4.2) in samples collected after 10 and 20 d of culture, and its disappearance later (Table 1), suggest its specific role in early organogenesis (*i.e.* the initiation of shoot organogenesis and meristemoids formation). The appearance of first basic isoform (pI 9) after 10 d and its increase in intensity after 40 and 60 d (Table 1) suggest its role during entire shoot organogenesis, being especially active in later stages. The observed switch between acidic and basic POD isoforms that occurred on the 40^{th} day (Table 1), suggest the role of specific isoforms in early and late organogenesis in *T. bellus*. Two basic POD isoforms (pI 8 - 9) are specific for late phases of organogenesis (Table 1). In other words presence or absence of those POD isoforms in a sample, alone or in combination with each other could be the marker of specific phases during *T. bellus* shoot organogenesis. Similarly differentiation of *Albizia odoratissima* shoot buds was also accompanied by the increase of POD activity and expression of new acidic POD isoform (Rajeswari and Paliwal 2008). These results could be the additional indication that different POD isoforms are involved in separate processes related to organogenesis (Kay and Basile 1987). Most SOD isoforms significantly decreased in intensity or even disappeared during *T. bellus* shoot organogenesis, while Fe-SOD is present in all samples (Table 1), suggesting its significance in all phases of *T. bellus* growth and shoot development. Acidic CuZn-SOD (pI 3.7) isoform is visible in all samples until shoot buds were formed (Table 1), meaning that its absence could be a marker of late phases of *T. bellus* shoot development. During indirect shoot organogenesis in *Acaranthophyllum sordidum* difference in SOD isoforms present in primary explants, calli and regenerated shoots were reported (Meratan *et al.* 2009). Polyphenol oxidase activity increased late during *T. bellus* shoot organogenesis (Table 1). Appearances of second basic PPO isoform associated with shoot buds formation, and with fully developed rosettes, suggest its role in later phases of

shoot organogenesis. During *Jatropa integerrima* shoot regeneration (Sujatha *et al*. 2000) high PPO activity was associated with formation of meristematic centres.

Our data suggest the role of specific CAT, POD, SOD and PPO isoforms in separate processes during *T. bellus* direct shoot organogenesis, pointing to two turning points: the initiation of shoot organogenesis and the shoot buds formation. The presences of two turning points during *T. bellus* shoot organogenesis, arising from experimental data, suggest fine regulation of H_2O_2 content. High SOD and no CAT and POD activity in primary explants, and decrease in SOD and increase in CAT and POD activity during *T. bellus* shoot organogenesis, suggest higher H_2O_2 content in intact plant than in tissue subjected to organogenesis. Described activity changes in H_2O_2-producing (SOD) and consuming enzymes (CAT and POD) suggest importance of maintenance of low H_2O_2 content during *T. bellus* shoot organogenesis.

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