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Antioxidant enzyme activities during *in vitro* morphogenesis of gladiolus and the effect of application of antioxidants on plant regeneration

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Abstract

Activity of antioxidant enzymes was evaluated during somatic embryogenesis and shoot organogenesis from cultured leaf segments of *Gladiolus hybridus* Hort. The effect of exogenous antioxidants on somatic embryogenesis and shoot organogenesis has also been monitored. Activity of superoxide dismutase (SOD) gradually increased during somatic embryogenesis, while activities of catalase (CAT) and peroxidase (POX) decreased. In contrast, increase in CAT and POX activity and a concomitant decrease in SOD activity were noted during shoot organogenesis. Exogenous application of antioxidants such as glutathione (GSH), α-tocopherol and ascorbate (AA) inhibited somatic embryogenesis but stimulated shoot organogenesis. The frequency of somatic embryogenesis increased with the addition of H_2O_2. However, H_2O_2 inhibited shoot organogenesis.

Additional key words: catalase, *Gladiolus hybridus*, peroxidase, reactive oxygen species, shoot organogenesis, somatic embryogenesis, superoxide dismutase.

Introduction

In plants, reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2^1) are constantly produced, however, uncontrolled production of ROS can cause cellular damage directly or through the formation of secondary toxic substances (Benson 2000). Plants have developed a complex antioxidant system to protect themselves against such oxidative damage (Larson 1988). Antioxidant protection system includes enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) as well as low molecular substrates like ascorbate (AA), glutathione (GSH) and α-tocopherol, which scavenge both radicals and their associated non-radical oxygen species.

The production of ROS has been associated with plant recalcitrance during *in vitro* culture (Benson 2000). Studies of lipid peroxidation in plant tissue culture demonstrate the accumulation of lipid peroxidase, 4-hydroxy-2-nonenol (HNE) and malondialdehyde (MDA) in cultures which have lost their regeneration potential (Benson 1992, Adams *et al.* 1999). Antioxidant stimulated improvement in growth and regeneration further support the relationship between oxidative stress and recalcitrance (Joy *et al.* 1988, Earnshaw and Johnson 1987, Creemers-Molenaar and Van Oort 1990). In recent years, there is an increasing interest to study the change of activities of antioxidant enzymes during different stages of shoot organogenesis (Franck *et al.* 1998, Chen and Ziv 2001) somatic embryogenesis (Cui *et al.* 1999) and *ex vitro* acclimation of regenerated plants (Van Huylenbroeck *et al.* 2000, Synková and Pospíšilová 2002). However, comparison of antioxidative enzyme status between shoot organogenesis and somatic embryogenesis of a species has not yet been made.

The objective of the present study was to compare the activities of antioxidative enzymes at various stages of shoot organogenesis and somatic embryogenesis of gladiolus. In addition the effect of exogenous application of antioxidants (ascorbic acid, glutathione and α-tocopherol) and pro-oxidant (H_2O_2) on the frequency of shoot organogenesis and somatic embryogenesis was also investigated.

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Abbreviations: AA - ascorbate; BA - 6-benzylaminopurine; CAT - catalase; GSH - glutathione; MS medium - Murashige and Skoog medium; NAA - 1-napthaleneacetic acid; POX - peroxidase; SOD - superoxide dismutase.

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Materials and methods

Plants and culture establishment: Corms of *Gladiolus hybridus* hort. cv. Weeding Boquet were sprouted on *Soilrite* mixture under 16-h photoperiod (irradiance of 60 μmol m^{-2} s^{-1}) at temperature of 25 °C. Sprouted shoots (7 to 10-d-old) were soaked in 5 % sodium hypochlorite with two to three drops of *Tween-20* for 15 min and washed thoroughly with sterile distilled water in a laminar flow transfer hood. Innermost leaves were then dissected out, cut into small pieces and cultured on Murashige and Skoog (1962) medium containing 2 mg dm^{-3} NAA, 3 % sucrose and 0.8 % agar for somatic embryo induction. Somatic embryos were induced at this treatment only from the basal cut ends (unpublished data). Other segments yielded the compact callus. Multiple shoots were regenerated by the transfer of compact callus to MS medium supplemented with 0.2 mg dm^{-3} NAA and 2 mg dm^{-3} BA (Sundaram and Dutta Gupta 2001). The pH of the medium was adjusted to 5.6 before autoclaving at 121 °C for 15 min. All the cultures were kept at 16-h photoperiod (irradiance of 60 μmol m^{-2} s^{-1}), temperature of 25 °C, and relative humidity of 55 %.

Antioxidant enzyme activities: Enzyme activities were estimated during somatic embryogenesis and shoot organogenesis at weekly intervals up to 4 weeks. During the first two weeks of somatic embryogenesis, analyses were performed with basal leaf sections, while analyses during early stages of shoot organogenesis were conducted with compact callus.

The sample of cultured tissues or leaves (0.5 g) was frozen in liquid nitrogen and ground to fine powder with a mortar and pestle. The powder was homogenized in 4 cm^3 of cold (4 °C) 0.05 M potassium phosphate buffer, pH 7.5 (Ranade and Feierabend 1991). The homogenate was centrifuged at 8000 *g* for 20 min at 4 °C. The supernatant was used for enzyme assays. Protein content of the crude enzyme extract was measured by Lowry method (Lowry *et al.* 1951).

Activity of SOD (EC 1.15.1.1) was assayed by monitoring the photochemical reaction of nitroblue tetrazolium (NBT) according to the method of Beyer and Fridovich (1987). The reaction mixtures contained 50 mM Hepes buffer (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO_3 (pH 10.4), 13 mM methionine, 0.025 % Triton X-100, 75 μM NBT, 2 μM riboflavin and 0.2 cm^3 aliquot of enzyme extract. The reaction mixture was irradiated (350 μmol m^{-2} s^{-1}) for 5 min and the absorbance was read at 560 nm against the non-irradiated blank. One unit activity of SOD was defined as the amount of enzyme, which caused 50 % inhibition in NBT reduction.

CAT (EC 1.11.1.6) activity was determined following the method of Klapheck *et al.* (1990) by measuring the decrease in absorbance at 240 nm due to H_2O_2 consumption. The reaction mixture (1 cm^3) contained 50 mM potassium phosphate buffer at pH 7.0, 0.036 % (m/m) H_2O_2 and the enzyme extract.

POX (EC 1.11.1.7) activity was assayed following the method of Kar and Mishra (1976). The assay mixture contained 2 cm^3 of 1.0 M pyrogallol, 0.3 cm^3 of 0.005 M H_2O_2 and 1 cm^3 of enzyme extract. The activity was estimated by measuring the absorbance at 420 nm.

Effect of antioxidants and pro-oxidants on somatic embryogenesis and shoot organogenesis: Antioxidants (ascorbate, glutathione and α-tocopherol) were filter sterilized and added to the embryo and shoot regeneration media at concentrations of 0.1, 0.5 and 1.0 mM. Similarly pro-oxidant H_2O_2 was added at concentrations of 0.05, 0.10 and 0.15 mM.

Statistics: Each treatment had 20 replications (culture tubes), each replication consisting of 3 explants, arranged in a completely randomized design. The percentage somatic embryogenesis/shoot organogenesis and number of somatic embryos/shoots per responding cultures were evaluated after 4 weeks of incubation. Data were subjected to analysis of variance (*ANOVA*) and means were separated by least significance difference (LSD) at 5 % probability level.

Results

Somatic embryogenesis in leaf segments of gladiolus proceeded via three developmental stages: *a*) swelling of the leaf explants, which occurred during the first 7 d of culture on MS medium supplemented with 2.0 mg dm^{-3} NAA; *b*) early callus formation arising from the cut ends of the explant during the 2nd week of culture, and *c*) appearance of cluster of somatic embryos along with callus within 3 weeks of incubation only from the basal part of the innermost leaves (details to be reported separately).

Compact callus produced from other segments were used for shoot organogenesis. Shoot buds were visible after 3 weeks of culture on MS medium supplemented with 0.2 mg dm^{-3} NAA and 2 mg dm^{-3} BA. Multiple shoots were developed after another 1 week.

The activity of SOD increased gradually in the early stages of somatic embryogenesis (Fig. 1*A*). SOD activity peaked on the 14th day of culture which was exactly the time when somatic embryos as tiny beads were detected. With further proliferation of somatic embryos there was a

fall in SOD activity. Contrary to somatic embryogenesis, a rapid decrease in SOD activity was observed (Fig. 1*A*) up to 21 d during shoot organogenesis. Interestingly, differentiation of shoot buds was noted around 3 weeks of culture.

CAT and POX activities were relatively high in the leaf explants at the time of culture initiation as compared to the compact callus used for shoot organogenesis. A gradual decrease in the activities of CAT and POX was observed during the induction and development of somatic embryos. The reduction was 1.85 and 1.76 fold, respectively, for CAT and POX after 4 weeks of culture. In contrast, CAT and POX activities increased considerably during shoot organogenesis (Fig. 1*B,C*). However, no significance difference was found between 14 and 21 d of culture which coincided with embryo and shoot emergence, respectively.

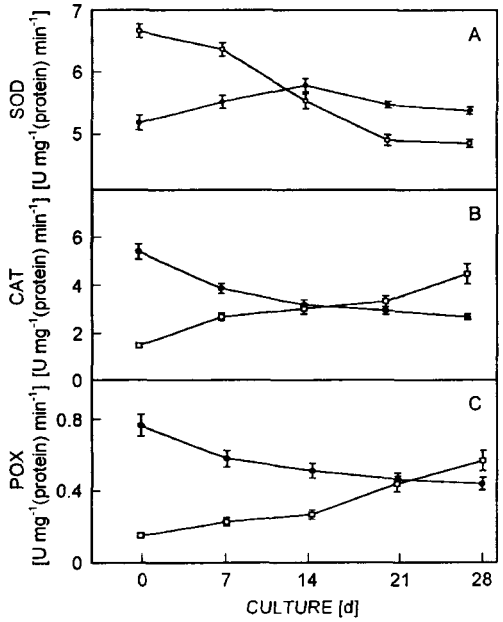

Fig. 1. Changes of SOD (*A*), CAT (*B*) and POX (*C*) activities during somatic embryogenesis (*closed circles*) and shoot organogenesis (*open squares*) of gladiolus.

In order to improve the regeneration frequency various antioxidants were added to the culture medium. Addition of antioxidants inhibited somatic embryogenesis (Table 1). The effect was more pronounced at higher concentrations. An opposite trend was observed during shoot organogenesis. Frequency of shoot organogenesis as well as number of shoots per responding culture was increased with the addition of antioxidants at 0.5 mM concentration (Table 1). However, higher concentrations were found to be inhibitory. Significant differences in the frequency of occurrence of embryogenesis/organogenesis and the number of somatic embryos/shoots were noted within the type and concentration of antioxidants without affecting the trends. Compared to α-tocopherol and AA, GSH was noted to be more effective.

In contrast to the observation made with antioxidants, H_2O_2 promoted somatic embryogenesis at 100 μM concentration (Table 1). H_2O_2 at 100 μM increased the frequency of somatic embryogenesis by about 18 %. Higher concentrations, however, inhibited the somatic embryo formation. A strong inhibitory response was obtained with all the concentrations of H_2O_2 during shoot organogenesis.

Table 1. Effect of antioxidants glutathione (GSH), α-tocopherol and ascorbate (AA) and pro-oxidant (H_2O_2) on somatic embryogenesis and shoot organogenesis from cultured leaf segments of gladiolus. Means ± SE, $n = 60$.

Compounds	Concentrations [mM]	Somatic embryogenesis [%]	Number of somatic embryos [responding culture^{-1}]	Shoot organogenesis [%]	Number of shoots [responding culture^{-1}]
Control	0.0	42.10 ± 1.58	35.06 ± 2.36	84.63 ± 1.21	14.96 ± 0.83
GSH	0.1	25.80 ± 1.02	12.96 ± 1.64	91.66 ± 1.11	19.86 ± 1.35
	0.5	12.83 ± 0.73	3.60 ± 0.54	80.03 ± 1.39	8.80 ± 0.66
	1.0	0.0 ± 0.0	0.0 ± 0.0	68.50 ± 1.52	5.80 ± 0.49
α-tocopherol	0.1	29.03 ± 1.46	9.36 ± 0.97	83.23 ± 1.54	15.13 ± 0.87
	0.5	15.70 ± 1.04	3.46 ± 0.54	87.66 ± 1.20	17.43 ± 1.08
	1.0	0.0 ± 0.0	0.0 ± 0.0	72.33 ± 1.63	8.83 ± 0.90
AA	0.1	32.15 ± 1.18	17.43 ± 1.11	88.96 ± 1.19	18.96 ± 1.15
	0.5	21.23 ± 1.24	7.96 ± 0.73	72.93 ± 1.69	13.50 ± 1.13
	1.0	5.00 ± 0.43	1.96 ± 0.24	5.13 ± 0.45	3.30 ± 0.50
H_2O_2	0.05	39.73 ± 1.25	32.50 ± 1.58	38.16 ± 1.62	5.56 ± 0.62
	0.1	48.92 ± 1.42	40.33 ± 2.18	15.83 ± 1.03	3.73 ± 0.44
	0.15	25.20 ± 1.37	18.53 ± 1.55	5.23 ± 0.44	3.16 ± 0.50
LSD		2.579	2.810	3.017	2.01

Discussion

In this study, SOD was chosen as it directly removes oxygen radicals, catalyzing the reaction $2O^{-}_{2} + 2H^{+} \rightarrow H_2O_2 + O_2$. The antioxidant role of CAT and POX was largely confined to the removal of H_2O_2 (Klapheck *et al.* 1990). As the results have shown, the acquisition of competence, induction and development of somatic embryos are associated with the gradual increase in SOD activity. However, there was a fall in CAT and POX activity. The present findings are consistent with previous studies on *Lycium barbarum* L. (Cui *et al.* 1999). It is important to consider that as SOD removes $O_2^{\cdot-}$, H_2O_2 is formed. It has been observed that low content of intracellular H_2O_2 induce and promote cell growth and somatic embryogenesis (Cui *et al.* 1999). It appears that during early days of somatic embryogenesis, cultured cells accumulated H_2O_2 which may have a stimulating effect on cell proliferation and somatic embryogenesis. The role of H_2O_2 as cellular messengers capable of inducing gene expression and protein synthesis has already been established (Willekens *et al.* 1994). But in latter stage excessive accumulation of H_2O_2 due to decreased activity of CAT and POX results in tissue browning and cell damage. Decreased activity of CAT and POX during dedifferentiation has also been observed in other plant systems and was correlated with the browning of tissue (Laukkanenn *et al.* 1999).

A different trend in the activity of antioxidant enzymes was observed during shoot organogenesis. Compared to embryogenic cultures, increased activity of CAT and POX was observed in organogenic cultures. This was associated with a decrease in SOD activity. Increase in CAT and POX and decrease in SOD as observed during shoot organogenesis were also reported in *Prunus* (Franck *et al.* 1995) and *Solanum* (Kumar and Knowles 1993). CAT and POX are known to play a role in growth and differentiation (Gaspar 1995) and their high activity could be correlated to the process of differentiation that occurred during shoot induction (Thakar and Bhargava 1999). The changes in antioxidant enzymatic activities appeared to be related to the mode of plant regeneration. Gradual accumulation of H_2O_2 as a result of increased SOD activity may induce and promote somatic embryogenesis. Conversely, scavenging of reactive oxygen species by CAT and POX induces shoot organogenesis.

Further study on the exogenous application of antioxidants and pro-oxidants adds to the body of evidence supporting such relationship. In the present investigation, addition of GSH, α-tocopherol and AA inhibited somatic embryogenesis in comparison to control both in terms of percent response and mean number of somatic embryos. Suppression of somatic embryos by the addition of antioxidants has been reported in wild *Daucus carota* (Earnshaw and Johnson 1987). On the other hand, the application of exogenous H_2O_2 showed enhanced somatic embryogenesis. A stimulatory role of H_2O_2 in somatic embryogenesis has also been reported by Cui *et al.* (1999). However, in the case of shoot organogenesis exogenous addition of antioxidants improved the frequency as well as number of shoots, whereas gradual inhibition in shoot regeneration was noted with the increase in concentration of H_2O_2. Stimulatory effect of antioxidant, particularly AA on shoot development has been observed in several *in vitro* systems (Joy *et al.* 1988, Stasolla and Yeung 1999).

In conclusion, the present work demonstrates for the first time that oxidative protection system during shoot organogenesis differs from that of somatic embryogenesis. Somatic embryogenesis prefers more stress environment than shoot organogenesis.

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