In vitro organogenesis and antioxidant enzymes activity in *Acanthophyllum sordidum*

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Abstract

The effect of various hormonal combinations on callus formation and regeneration of shoot and root from leaf derived callus of *Acanthophyllum sordidum* Bunge *ex* Boiss. has been studied. Proteins and activity of antioxidant enzymes were also evaluated during shoot and root organogenesis from callus. Calli were induced from leaf explants excised from 30-d-old seedlings grown on Murashige and Skoog medium containing 4.52 μM 2,4-dichlorophenoxyacetic acid + 4.65 μM kinetin. Maximum growth of calli and the most efficient regeneration of shoots and roots occurred with 2.69 μM 1-naphthalene acetic acid (NAA), 2.69 μM NAA + 4.54 μM thidiazuron and 2.46 μM indole-3-butyric acid. Protein content decreased in calli and increased significantly during regeneration of shoots from callus. Superoxide dismutase activity decreased in calli comparing to that of seedlings, then increased in regenerated shoots and roots. High catalase activity was detected in seedlings and regenerated shoots, whereas high peroxidase activity was observed in calli and regenerated roots.

Additional key words: callus, *Caryophyllaceae*, catalase, peroxidase, protein, regeneration, superoxide dismutase, tissue culture.

Introduction

Acanthophyllum is a genus with more than 38 species in Iran. *A. sordidum* Bunge *ex* Boiss. is a tetraploid (2n=60) medicinally valuable salt and drought resistant plant of family *Caryophyllaceae*. The plant is a shrubby or herbaceous with woody rootstock. Over-exploitation of the natural population for medicinal use and the lack of systematic efforts at cultivation call the need for *in vitro* culture. Hence, the present study was undertaken in an effort to meet the increasing demands of *A. sordidum* using *in vitro* propagation techniques.

The production of reactive oxygen species (ROS) has been associated with plant recalcitrance during *in vitro* culture (Benson 2000). The role of ROS and antioxidant enzymes during organogenesis and somatic embryogenesis in some species has been demonstrated (*e.g.* Yang *et al.* 1993, Kairong *et al.* 1999, Tian *et al.* 2003, Tang and Newton 2005). In recent years, there has been a growing interest to study the changes of antioxidant enzymes during organogenesis (*e.g.* Chen and Ziv 2001, Gupta and Datta 2003) Moreover, there are several reports of *in vitro* organogenesis in plant species (*e.g.* Chen *et al.* 2006, Khanna *et al.* 2006, Radhika *et al.* 2006, Selvaraj *et al.* 2006).

In this study, we established a system for shoot and root organogenesis from callus and surveyed the changes of proteins and antioxidant enzyme activities in seedling, callus and regenerated shoot and root.

Materials and methods

Plants and culture conditions: Seeds of *Acanthophyllum sordidum* Bunge *ex* Boiss. collected from nature were surface sterilized in 90 % ethanol for 1 min followed by 15 % NaHClO for 10 min. They were

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Abbreviations: MS - Murashige and Skoog; 2,4-D - 2,4-dichlorophenoxy acetic acid; Kin - kinetin; NAA - 1-naphthalene acetic acid; IBA - indole-3-butyric acid; TDZ - thidiazuron; BA- benzyl adenine; IAA - indole-3-acetic acid; SOD - superoxide dismutase; CAT - catalase; POX - peroxidase; ROS - reactive oxygen species; SDS - sodium dodesyl sulfate; PAGE - polyacrylamide gel electrophoresis; Zea - zeatin.
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rinsed three times in sterile distilled water and cultured on Murashige and Skoog (1962; MS) medium containing 8 % agar and 3 % sucrose under 16-h photoperiod (white fluorescent lamps; irradiance of 46 µmol m^{-2} s^{-1}) and day/night temperature of 25/20 °C. Calli were induced from leaf explants excised from 30-d-old seedlings (Fig. 1*A*) grown on MS medium containing 4.52 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.65 µM kinetin (Kin).

The growth regulator combinations tested included 4 combinations of 2,4-D (2.26 - 9.05 µM) + Kin (2.32 - 4.65 µM); 2 combinations of 1-naphthalene acetic acid (NAA; 2.69 µM) + thidiazuron (TDZ; 0 and 4.54 µM); 4 combinations of NAA (2.69 and 5.37 µM) + zeatin (Zea; 2.28 and 4.56 µM); 2 combinations of indole-3-butyric acid (IBA; 2.46 µM) + zeatin (0 and 9.12 µM) and 2 combinations of indole-3-acetic acid (IAA; 2.85 and 5.71 µM) + benzyladenine (BA; 0 and 4.44 µM).

For initiation of shoots and roots, 45-d-old calli were subcultured on MS medium containing different concentrations of 2,4-D, NAA, IBA and IAA either alone or in combinations with different concentrations of Kin, TDZ, zeatin and BA (Table 1). All chemicals used were of analytical grade (*Sigma-Aldrich* and *Merck*). Experiments were carried out in 15 replicates and each experiment was repeated three times. The average number of calli and regenerated shoots and roots induced per Petri dish was recorded after 30 d of culture.

Antioxidant enzyme activities: For estimation of total protein content and enzyme activity, homogenate of seedlings (as control), calli, shoots and roots was extracted with ice-cold 0.5 M Tris-HCl (pH 6.8) buffer. The extracts were centrifuged at 16 000 *g* for 30 min at 4 °C and resulting supernatants were frozen in liquid nitrogen, kept in -70 °C and used for protein determination and enzyme assays. A high-speed centrifuge (*J2-21M, Beckman*, Palo Alto, USA) and UV-visible spectrophotometer (*UV-160, Shimadzu,* Tokyo, Japan) with 10 mm matched quartz cells were used for centrifugation of the extracts and determination of the absorbance, respectively. The protein content was determined according to the method of Bradford (1976) using bovine serum albumin as a standard. Superoxide dismutase (SOD; E.C. 1.15.1.1) activity was estimated by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm as described by Giannopolitis and Ries (1977) in a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.50), 13 mM methionine, 75 µM nitro blue tetrazolium, 2 µM riboflavin, 0.1 mM EDTA and 0.02 cm^3 of enzyme extract. Catalase (CAT; E.C. 1.11.1.6) activity was assayed from the rate of H_2O_2 decomposition as measured by the decrease of absorbance at 240 nm, following the procedure of Arrigoni *et al*. (1992). The reaction mixture contained 0.625 cm^3 50 mM sodium phosphate buffer (pH 7.0), 0.075 cm^3 H_2O_2 (3 %), and 0.03 cm^3 enzyme extract. Peroxidase (POX; E.C. 1.11.1.7) activity was measured according to the method of Abeles and Biles (1991). The reaction mixture contained 2 cm^3 of 0.2 M acetate buffer (pH 4.8), 0.2 cm^3 H_2O_2 (3 %), 0.2 cm^3 40 mM benzidine and 0.02 cm^3 enzyme extract. The increase in absorbance was recorded at 530 nm.

Gel electrophoresis: For determination of protein profiles in seedlings, calli, shoots, and roots discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) using 12 % acrylamide concentration. For isoenzymes assay, native-PAGE was carried out by a modified method of Davis (1964) with a 10 % (SOD and POX) and 8 % (CAT) acrylamide gels at 4 °C without SDS and mercaptoethanol. A mini vertical electrophoresis unit (*model LKB, Bromma,* Stockholm, Sweden) was used. Activity staining for SOD was determined by method of Wendel and Weeden (1989). Gels were briefly washed in distilled water followed by incubation in 0.2 M Tris-HCl (pH 8.0) containing 4 % riboflavin, 4 % EDTA and 20 % NBT for 40 min. Then the gels were placed under one 15-W fluorescent tubes and shaked slowly till white bands appeared in violet background. CAT activity was detected using the method of Woodbury *et al.* (1971). The gels were incubated in 5 mM H_2O_2. After 10 min the gels were washed with distilled water and stained with a reaction mixture containing 2 % (m/v) ferricyanide and 2 % ferric chloride till yellow bands on dark green back ground appeared. Electrophoretic pattern of POX was obtained by staining the gels by benzidine according to Van Loon (1971). The gels immersed in 0.2 M acetate buffer (pH 4.8) containing 3 % H_2O_2 and 4 % benzidine in 50 % methanol at room temperature till the brown colour.

Statistical analysis: Each experiment was repeated at least three times. Analysis of variance was conducted using one-way *ANOVA* test using *SPSS 9.01* for *Microsoft Windows* and means were compared by Duncan tests at the 0.05 level of confidence.

Results and discussion

Callus was induced from leaf explant on MS medium containing 4.52 µM 2,4-D + 4.65 µM Kin. In order to induce organogenesis, 45-d-old calli (Fig. 1*B*) were transferred to MS medium containing different hormone concentrations. Among the 14 hormonal combinations tested, the highest frequency of callus formation was achieved by 2.69 µM NAA (Table 1). This is the first report on callus production in *A. sordidum*. Callus

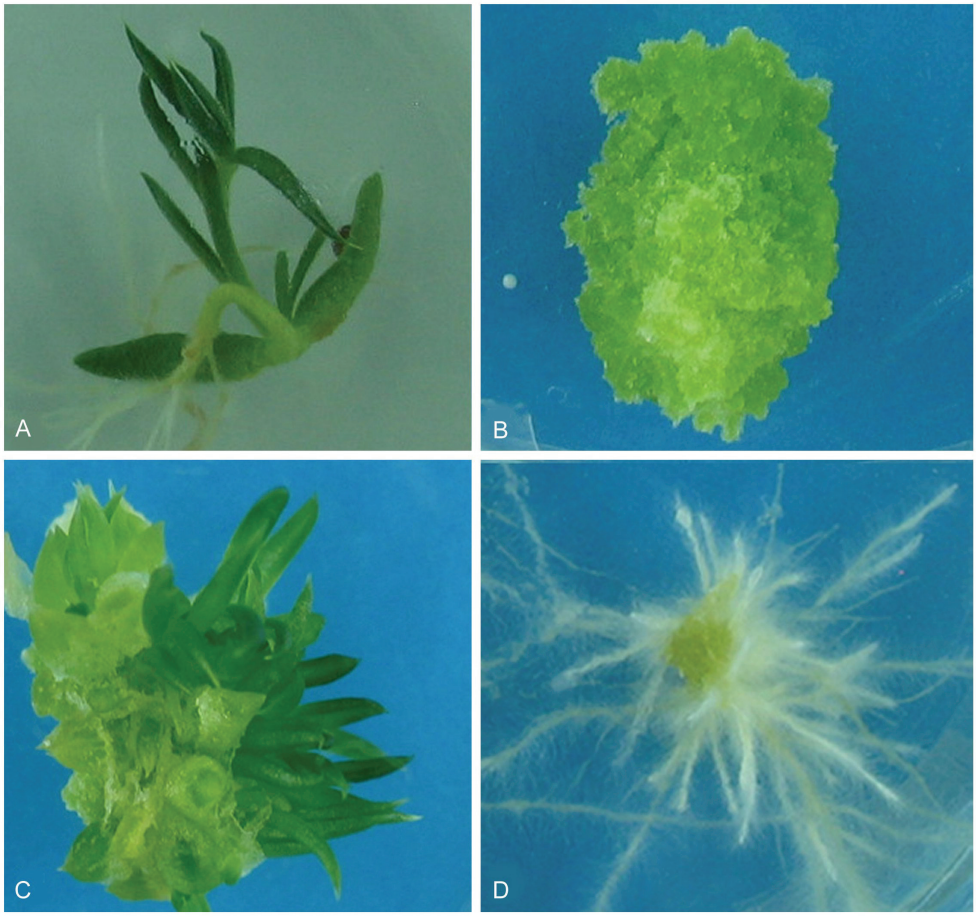

Fig. 1. Shoot and root regeneration from callus of *A. sordidum* on MS medium: *A* - seedling; *B* - callus induced from leaflet explant on medium supplemented with 4.52 µM 2,4-D + 4.65 µM Kin; *C* - shoot regeneration through callus on medium with 2.69 µM NAA + 4.54 µM TDZ; *D* - root regeneration through callus on medium supplemented with 2.46 µM IBA.

Table 1. Effect of various concentrations [µM] and combinations of growth regulators on callus formation and regeneration of shoot and root from organogenic callus of *A. sordidum*. Means ± SE, $n = 3$; values marked with different letters are significantly different according to DMRT at $P < 0.05$.

	Callus [%]	Shoot [%]	Root [%]
2,4-D 2.26+Kin 2.32		32.13±1.92d	
2,4-D 4.52+Kin 2.32		6.56±1.22e	
2,4-D 4.52+Kin 4.65	65.00±2.28b		
2,4-D 9.05+Kin 4.65	38.46±2.09c		
NAA 2.69	79.00±0.65a		
NAA 2.69+TDZ 4.54		78.62±2.18a	
NAA 2.69+Zea 2.28	31.74±2.21d		
NAA 2.69+Zea 4.56		32.76±1.83d	
NAA 5.37+Zea 2.28		42.21±1.61c	
Zea 4.56		59.76±1.23b	
IBA 2.46			84.24±2.23a
IBA 2.46+Zea 9.12			71.44±2.28b
IAA 2.85			41.87±2.65c
IAA 5.71+BA 4.44			17.70±1.93d

Table 2. Protein content [mg g^{-1}(f.m.)] and activity of SOD, CAT and POX [U mg^{-1}(protein)] in seedlings, calli and regenerated shoots and roots of *A. sordidum*. Means ± SE, $n = 3$; values marked with different letters are significantly different according to DMRT at $P < 0.05$.

	Protein	SOD	CAT	POX
Seedlings	11.31±0.23a	21.98±0.93a	24.79±0.53b	9.52±1.31d
Calli	2.08±0.05c	11.64±0.66c	1.86±0.27c	35.66±0.67a
Shoots	7.57±0.08b	18.20±1.19b	36.29±0.69a	17.04±1.50c
Roots	2.39±0.06c	14.80±0.86d	1.02±0.15c	25.99±1.47b

production may be important for studies of indirect morphogenesis (*e.g.* somatic embryogenesis) or for studies of production of secondary metabolites.

Shoot formation (Fig. 1*C*) from callus was induced by 6 out of 14 hormonal combinations (Table 1). Frequency of shoot formation varied between 6.56 - 78.62 %, the lowest frequency was observed on medium containing 4.52 µM 2,4-D + 2.32 µM Kin and the highest with 2.69 µM NAA + 4.54 µM TDZ. These results are in

agreement with the findings of Chevreau *et al.* (1989) and Radhika *et al.* (2006). Therefore, TDZ which is known for induction of adventitious shoots and proliferation of axillary shoots in woody species (Huetteman and Preece 1993) holds promise for a perennial like *A. sordidum*. Moreover, while 2.69 μM NAA + 2.28 μM Zea prevented callus differentiation, 2.69 μM NAA + 4.56 μM Zea induced shoot formation.

Maximum percentage of root formation (Fig. 1*D*) from callus (84.57 %) was observed on medium supplemented with 2.46 μM IBA (Table 1). This result is in agreement with the findings of Dhar *et al.* (2000) on *Pittosporum napaulensis* and Agarwal and Sardar (2006) on *Cassia angustifolia*, where IBA was found better than NAA and IAA to induce the formation of maximum number of roots. The promotion of rooting from callus by IBA has also been reported in many other plant species (*e.g.* Saritha *et al*. 2002, Soniya and Das 2002, Soniya and Sujitha 2006, Zhang *et al*. 2004). Regeneration of roots also occurred on some concentrations of IAA, but with less frequency.

Protein content was low in callus and increased in regenerated shoot (Table 2). Calli secreted antioxidant enzymes such as SOD and CAT during callus differentiation. Tian *et al.* (2003) demonstrated that antioxidant enzymes were involved in the process of shoot organogenesis in strawberry callus. Activity of SOD increased during organogenesis, while activity of POX decreased (Table 2). Increase in CAT activity was noted only during shoot organogenesis. The greater increase in CAT activity of regenerated shoot than in callus and regenerated root may suggest its effective scavenging mechanism to remove H_2O_2 produced in regenerated shoot. Increase in CAT activity during shoot organogenesis is in agreement with the result of Gupta and Datta (2003) in *Gladiolus hybridus* Hort. CAT and POX are known to play a role in growth and differentiation (Gaspar 1995, Molassiotis *et al.* 2004) and their high activity could be correlated to the process of differentiation that occurred during shoot or root induction (Thakar and Bhargava 1999). Moreover, although both of CAT and POX enzymes are involved in elimination of H_2O_2, owing to high reaction rate but low affinity for H_2O_2, CAT only remove the bulk of H_2O_2 (Willekens *et al.* 1997), whereas, POX because of high affinity for H_2O_2, eliminated small amounts of H_2O_2 (Dat *et al.* 2000). Therefore, higher activity of CAT in regenerated shoot and POX in regenerated root could be due to higher and lower content of H_2O_2 in regenerated shoot and root, respectively.

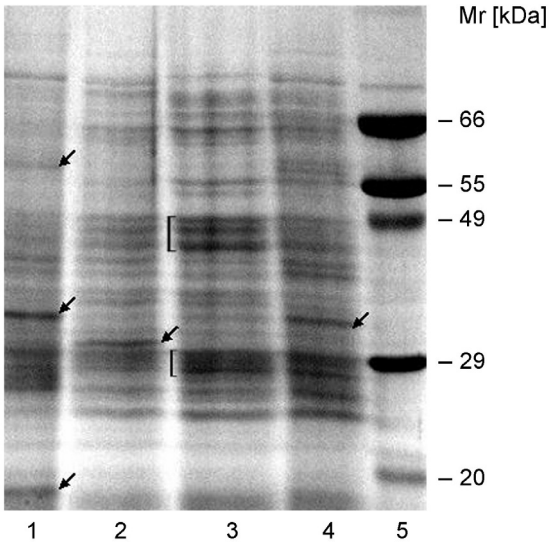

Fig. 2. SDS-PAGE pattern of proteins during callus induction and indirect organogenesis of *A. sordidum*: seedling (1), regenerated root (2), callus (3), regenerated shoot (4) and molecular mass marker (5). Equal amounts of protein (40 μg) were loaded in each lane. *Arrows* indicate the new and affected bands.

According to the SDS-PAGE analysis (Fig. 2), several proteins existed distinctively in callus and are absent in the other samples, or their amounts are lower than in calli. In contrast, high amount of a 33 kDa protein lower amount of a 58 kDa protein were detected only in seedlings and regenerated shoots and accumulation of the 14.2 and 30 kDa proteins was noted only in seedling and regenerated roots. Accumulation of 30 kDa protein in regenerated roots suggested that this polypeptide may play a role in root regeneration. The organogenesis from the callus is a very complex process, accompanied with a variety of genes expression and protein synthesis (Tian *et al.* 2003) where H_2O_2 can play a role (Willekens *et al.* 1994). So, we suggested that the endogenous H_2O_2 is capable of inducing gene expression and protein synthesis, thus regulating the organogenesis also in *A. sordidum*.

According to non-denaturing PAGE, six SOD isoforms were detected (Fig. 3*A*). SOD 1 was detected only in seedlings and regenerated shoots. SOD 2 was detected in both calli (higher amount) and regenerated roots. SOD 3 - 5 were differently expressed in seedlings, calli and regenerated shoots and roots. SOD 6 was detected in all the specimens but the amount in roots was higher than that of the others. One CAT isoform (CAT 1) was detected only in the seedlings and regenerated shoots (Fig. 3*B*). Five POX isoforms (POX 1 - POX 5) were detected (Fig. 3*C*). POX 3 was detected only in regenerated shoots. POX 5 activity was highest in the calli and then decreased during root and shoot regeneration. According to Gaspar *et al*. (1982) the POX exists in multiple isoforms that are developmentally regulated and highly responsive to exogenous stimuli. Moreover, the detected SOD, CAT and POX isoforms confirmed the spectrophotometric determinations.

In conclusion, the present work demonstrates for the first time the regeneration of shoot and root from callus and the involvement of oxidative protection system during organogenesis from callus cultures of an important medicinal plant *A. sordidum*.

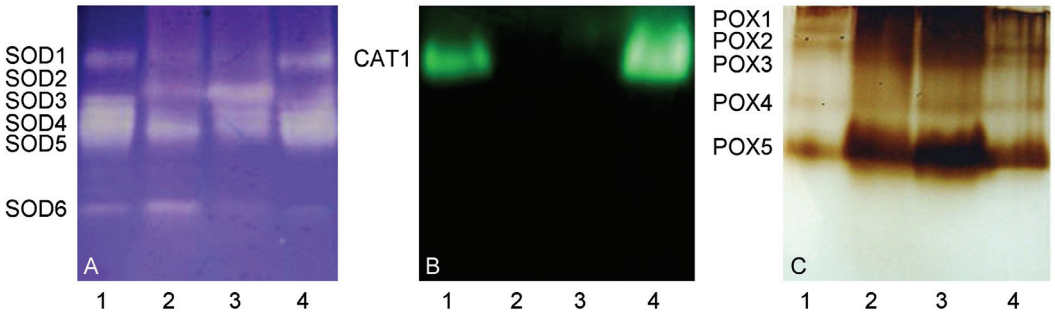

Fig. 3. Activity staining for SOD (*A*), CAT (*B*) and POX (*C*) during callus induction and indirect organogenesis of *A. sordidum*: seedling (1), regenerated root (2), callus (3), and regenerated shoot (4). Equal amounts of protein (40 μg) were loaded in each lane.

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