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Pathways of ROS homeostasis regulation in *Mesembryanthemum crystallinum* L. calli exhibiting differences in rhizogenesis

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Abstract A comparison of the hydrogen peroxide (H_2O_2) content, proline and betacyanin concentration and activities of some antioxidant enzymes (catalase, superoxide dismutase, guaiacol and ascorbate peroxidases) was made in *Mesembryanthemum crystallinum* L. calli differing in rhizogenic potential. Callus was induced from hypocotyls of 10-day-old seedlings on a medium containing 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.2 mg l^{-1} kinetin, which was either supplemented with 40 mM NaCl (CIM-NaCl medium) or did not contain any salt (CIM medium). The callus obtained on CIM-NaCl was rhizogenic, whereas the callus induced on the medium without salt was non-rhizogenic throughout the culture. The rhizogenic callus differed from the non-rhizogenic callus in lower betacyanin and H_2O_2 content, but the rhizogenic callus displayed a higher proline level. The activity of H_2O_2 scavenging enzymes, such as catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POD), was markedly higher in the rhizogenic callus than in the non-rhizogenic callus, but the total activity of superoxide dismutase (SOD) was higher in the non-rhizogenic callus than in the rhizogenic callus. Aminotriazole (CAT inhibitor) and diethyldithiocarbamate (SOD inhibitor) were added solely to the CIM and CIM-NaCl media to manipulate the concentration of reactive oxygen species (ROS) in the cultured tissues. Both CAT and SOD inhibitors brought about an increase in H_2O_2 content in calli cultured on CIM-NaCl and the loss of rhizogenic potential. Conversely, the addition of inhibitors to the medium without salt led to a decrease in H_2O_2 content. This corresponded with a significant decrease in the endogenous concentration of betacyanins, but did not change the lack of rhizogenic ability.

Keywords Adventitious rooting · Betacyanins · Cell reprogramming · Ice plant · Proline · Reactive oxygen species

Abbreviations
APX	Ascorbate peroxidase (EC 1.11.1.11)
AT	3-Amino-1,2,4-triazole
BSA	Bovine serum albumine
CAM	Crassulacean acid metabolism
CAT	Catalase (EC 1.11.1.6)
DDTC	Diethyldithiocarbamate
DTT	Dithiothreitol
2,4-D	2,4-Dichlorophenoxyacetic acid
EDTA	Ethylenediamine tetraacetic acid
CIM	Callus induction medium
GM	Germination medium
MS	Murashige and Skoog medium
NAA	Naphtaleneacetic acid
NBT	Nitro blue tetrazolium
PAGE	Polyacrylamide gel electrophoresis
PAR	Photosynthetically active radiation
POD	Guaiacol peroxidase (EC 1.11.1.7)
RH	Relative humidity
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SIM	Shoot induction medium
SOD	Superoxide dismutase (EC 1.15.1.1)
TEMED	N,N,N′,N′-tetramethylethylenediamine

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TRICINE N-tris[hydroxymethyl]methylglycine
TRIS   Tris(hydroxymethyl)aminomethane

Introduction

Reactive oxygen species (ROS) are known to be an intrinsic signal in plant development (Vranová et al. 2002; Ślesak et al. 2007). It has already been demonstrated that oxidative stress is involved in regeneration and morphogenic processes in in vitro culture (Kapur et al. 1993; Roubelakis-Angelakis 1993; Papadakis et al. 2001; Konieczny et al. 2008; Macedo et al. 2009; Kalra and Babbar 2010; Abbasi et al. 2011; Petřivalský et al. 2011). Oxidative stress results from an imbalance between ROS generation and the antioxidant capacity of cells. The level and type of ROS, e.g. superoxide radical ($O_2^{.-}$), hydrogen peroxide ($^.H_2O_2$) or hydroxyl radical ($^.OH$), determine plant cell response. At high concentrations, ROS can elicit severe oxidative damage. However, at low concentrations ROS act as signalling molecules engaged in mechanisms of induction of adaptive responses (Vranová et al. 2002). Several antioxidant enzymes, e.g. superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT), are involved in the regulation of the ROS level. SOD (EC 1.15.1.1) is a metalloenzyme which plays a key role in protecting against ROS by converting the superoxide radical to hydrogen peroxide and oxygen. In *Mesembryanthemum crystallinum* leaves, three isoforms of SOD were characterized: MnSOD, FeSOD and CuZnSOD (Miszalski et al. 1998). Catalase (EC 1.11.1.6) and peroxidases (EC 1.11.1.7) take part in the conversion of hydrogen peroxide to water and molecular oxygen. In green tissues catalase acts mainly as a sink for H_2O_2 during photorespiration (Ślesak et al. 2007). Ascorbate peroxidase (APX, EC 1.11.1.11) is the most important peroxidase in H_2O_2 detoxification, catalyzing the reduction of H_2O_2 with the use of reducing power of ascorbate (Noctor and Foyer 1998). Guaiacol peroxidase (POD) also participates in hydrogen peroxide removal during processes such as biosynthesis of lignin, plant development and organogenesis as well as senescence and responses to wounding and pathogens (Matamoros et al. 2003).

Mesembryanthemum crystallinum L. is a facultative halophyte that tolerates NaCl in concentrations up to 500 mM. Salt tolerant plants exhibit some general physiological characteristics, such as high cellular content of Na^+ resulting in a declined K^+/Na^+ ratio and a high proline level (Kumar and Sharma 1989). Salinity stress, similarly as other environmental stress factors, leads to overproduction of ROS and induction of oxidative stress.

In vitro tissue culture models can be a useful tool in studying the oxidative stress responses independently of regulatory pathways occurring at the whole plant level. *M. crystallinum* can be cultivated in vitro (Meiners et al. 1991; Wang and Lüttge 1994; Yen et al. 1997; Cushman et al. 2000; Ślesak and Miszalski 2003; Ślesak et al. 2003), which allows to control the induction of regeneration in the cultured explants of this plant (Libik et al. 2005) and allows to process plant morphogenesis by manipulating the level of some signaling molecules, such as ROS.

In previous research we investigated changes in the level of H_2O_2 and activity of some antioxidant enzymes during rhizogenesis and somatic embryogenesis in the callus of *M. crystallinum*, which were induced on callus induction medium (CIM) containing salt. Differences in the antioxidant system between calli exhibiting different morphogenic potential (rhizogenic or embryogenic) were found, and it was suggested that modification in the oxidative events might be linked to different metabolic pathways accompanying various morphogenic processes (Libik et al. 2005). In the work demonstrated in this paper we focused on the induction of rhizogenesis and accumulation of betacyanins in the callus culture on a callus-inducing medium containing salt (CIM-NaCl) and callus cultured on a callus-inducing medium without salt addition (CIM). We observed that CIM callus and CIM-NaCl callus differed from each other in their ability to regenerate roots and their ability to accumulate betacyanins—the red-violet pigments belonging to betalains. Betacyanins are accumulated, instead of anthocyanins, in high amounts in the vacuoles of different plant organs (Voght et al. 1999; Wybraniec and Mizrahi 2002). In the *M. crystallinum* plant betacyanins are present at the adult and flowering growth phases in the epidermal bladder cells; in the flowers and young leaves. However, the pigments are absent in the juvenile growth phase, but might be induced by some stress factors, such as salinity and high light (Ibdah et al. 2002). Betacyanins have recently gained more attention due to their antioxidant properties and their possible protective role in several human degenerative diseases (Escribano et al. 1998). However, hitherto little attention has been paid to the stress-induced betacyanins synthesis in the in vitro culture of plants.

The results of our experiments allow us to discuss the assumption that in two types of calli: (1) rhizogenic, but lacking the ability to accumulate betacyanins, and (2) non-rhizogenic, but accumulating high amounts of betacyanins; ROS homeostasis is controlled by two distinct pathways. Inhibitors of antioxidant enzymes were used to test the possible role of ROS balance in orchestrating processes taking part during the in vitro culture of *M. crystallinum* callus. Diethyldithiocarbamate (DDTC)-mediated inhibition of SOD and aminotriazole (AT)-mediated inhibition of

CAT were used as tools modifying ROS concentration due to changes in ROS production and removal processes.

Materials and methods

Plant material

Mesembryanthemum crystallinum L. seeds were obtained from plants growing under greenhouse conditions. The seeds were surface sterilised by immersion in 70% (v/v) ethanol for 2 min, then in a commercial bleach solution diluted with water (1:2; v/v) for 10 min, and finally in a more diluted bleach solution (1:10; v/v) for 5 min. Following sterilisation, the *M. crystallinum* seeds were rinsed three times with sterile, distilled water and then placed into 100 ml Erlenmeyer flasks (20–30 seeds per flask) containing a solid germination medium (GM; 2.15 g l^{-1} MS salts (Murashige and Skoog 1962), 100 mg l^{-1} myo-inositol, 0.4 mg l^{-1} thiamine-HCl, 67 mg l^{-1} adenine hemisulphate, 2 mg l^{-1}, sucrose and 5 g l^{-1} agar (Difco-Bacto, Detroit), pH 5.7. Flasks containing the seeds were placed in a growth chamber for germination at 25/20 °C under a 16/8 h light/dark with light provided by cool-fluorescent light, 150–200 μmol $m^{-2}s^{-1}$.

In vitro culture conditions

To induce callus formation, hypocotyl explants (about 4 mm in length) were excised from 10-day-old seedlings and were inoculated into 100 ml Erlenmeyer flasks containing 20 ml solid medium (4.3 g l^{-1} MS salts, 100 mg l^{-1} myoinositol, 10 mg l^{-1} thiamine HCl, 1 mg l^{-1} nicotinic acid, 1 mg l^{-1} pyridoxine HCl, 0.2 mg l^{-1} kinetin, 1 mg l^{-1} 2,4-D, 30 mg l^{-1} sucrose) with NaCl (4.64 g l^{-1}, CIM-NaCl) or without NaCl (CIM) (ten explants/flask). Calli (4-week-old) obtained from the two different hypocotyl cultures (CIM-NaCl and CIM) were subcultured on a fresh medium with the same composition (CIM-NaCl and CIM) but supplemented with 5 mM DDTC (CIM-NaCl DDTC, CIM DDTC) or 2.5 mM AT (CIM-NaCl AT, CIM AT). These cultures were maintained for 2 weeks.

Biochemical analysis

Analyses were performed on extracts isolated from 6-week-old calli described as: CIM, CIM DDTC, CIM AT and CIM-NaCl, CIM-NaCl DDTC, CIM-NaCl AT calli.

Protein isolation

To isolate fractions of soluble proteins, the plant material was homogenised (1 g fresh weight) at 4 °C with a mortar in a 2.5 ml homogenisation buffer (17.9 g l^{-1} TRICINE, 0.74 g l^{-1} MgSO$_4$, 0.155 g l^{-1} DTT, 1.14 g l^{-1} EDTA, adjusted with 1 M TRIS to pH 8.0). Non-soluble material was removed by centrifugation for 3 min at 3,000*g*.

Determination of protein content

The protein concentration was determined according to Bradford (1976) using the BioRad protein assay.

Analysis of superoxide dismutase (SOD) activity on gel after native PAGE

To determine the activity of SOD, fractions of soluble proteins were analysed. These were isolated as described above and separated using native PAGE at 4 °C and 180 V in the Laemmli (1970) buffer system without sodium dodecyl sulfate (SDS). SOD bands were visualised on 12% polyacrylamide gels using the activity staining procedure described by Beauchamp and Fridovich (1971), i.e. the gels were incubated in a staining buffer [potassium phosphate buffer, pH 7.8, containing 0.0068 g l^{-1} KH$_2$PO$_4$, 0.0175 g l^{-1} Na$_2$HPO$_4$, 0.372 g l^{-1} EDTA, 31% (w/v) TEMED, 7.5 mg l^{-1} riboflavin and 0.2 g l^{-1} NBT] for 30 min in the dark at room temperature, then exposed to white light until the SOD activity bands became visible.

Densitometric analysis

The gel images were analysed using BIOPRINT ver. 99 computer software (Vilber-Lourmat, France). The activities of all isoforms were determined in arbitrary units corresponding to the area under the densitometric curve.

Spectrophotometric analysis

Betacyanin content

The betacyanin level was measured according to the method described by Schliemann et al. (2001). Extracts were prepared from 0.5 g of plant material homogenised in 50% aqueous methanol (2 ml). Then the extracts were centrifuged in an Eppendorff centrifuge at 16,000*g* for 5 min at 4 °C. To determine the betacyanin content, absorbance of the supernatant was measured at 540 nm. The concentration of betacyanin was calculated using the absorbance coefficient 56.6×10^6 cm^{-2} mol^{-1}. Assays were performed at least in triplicates.

Hydrogen peroxide determination

The endogenous H$_2$O$_2$ concentration was measured according to the modified method described previously by

Brennan and Frenkel ([1977]). Hydrogen peroxide was extracted by homogenisation of 0.5–1 g of tissue in 2 ml of cold acetone. After centrifugation (5 min at 12,000g) the pellet was discarded and 0.5 ml of the extract was collected. Titanium reagent (50 μl of 20% titanium tetrachloride in concentrated HCl, v/v) was added to 0.5 ml of the extract, followed by the addition of 0.1 ml of NH_3 aq. (25%) to precipitate the peroxide-titanium complex. After 5 min of centrifugation at 10,000g, the supernatant was discarded and the precipitate was repeatedly washed in 1 ml of acetone and centrifuged again for 5 min at 10,000g. The precipitate was solubilised in 1 ml of 1 M H_2SO_4 and brought to a final volume of 2 ml. Absorbance of the obtained solution was read at 415 nm against a water blank. The concentration of H_2O_2 in the extract was determined by comparing the absorbance against a standard curve representing the titanium-H_2O_2 complex, over the range from 0 to 20 μmol ml^{-1}. All H_2O_2 measurements were normalised to a fresh weight of tissue.

Activity of hydrogen peroxide scavenging enzymes

Catalase (CAT) activity was determined after protein isolation from 1 g of frozen tissue in 2 ml of 300 mM Tricine buffer pH 8.0, containing 3 mM $MgSO_4$ and 3 mM EGTA and 1 mM DTT. The extract was then centrifuged at 3,000g at 4 °C and the supernatant was collected. CAT activity was measured according to the method described by Aebi ([1984]). The disappearance of H_2O_2 [initial concentration: 0.04% (v/v) H_2O_2] in a phosphate buffer (50 mM KH_2PO_4, 50 mM Na_2HPO_4 pH 7.0) was monitored at 240 nm. Enzyme activity was defined as 1 μmol of H_2O_2 decomposed per minute. For calculation, the absorbance coefficient 43 l M^{-1} cm^{-1} was used.

Guaiacol peroxidase (POD) activity was determined according to Bergmeyer ([1974]), after homogenisation of 0.1 g of frozen tissue in 1 ml 300 mM potassium phosphate extraction buffer, pH 7.0, containing 1 mM EDTA. The extract was centrifuged at 10,000g at 4 °C for 3 min. The reaction was run for 5 min at 25 °C in a 1 ml cuvette with 50 μl of purified extract in 300 mM potassium phosphate buffer, pH 6.1, in the presence of 8.42 mM guaiacol and 2.10 mM H_2O_2. The conversion of guaiacol to tetraguaiacol was monitored at 470 nm, and POD activity was calculated using the absorbance coefficient 26,600 l M^{-1} cm^{-1}.

Ascorbate peroxidase (APX) activity was determined according to Nakano and Asada ([1981]), using 0.1 g of frozen tissue that was homogenised in 1 ml of 300 mM potassium phosphate buffer, pH 7.0, with the addition of 1 mM EDTA and 5 mM ascorbate. The extract was then centrifuged at 10,000g at 4 °C for 3 min. The reaction was run for 2 min at 25 °C, in a 1 ml cuvette with 50 μl extract in 300 mM KPO_4, pH 7.0, in the presence of 0.75 mM ascorbate and 2 mM H_2O_2. The conversion of ascorbate into dehydroascorbate was monitored at 290 nm and the APX activity was calculated using the absorbance coefficient 2,800 l M^{-1} cm^{-1}.

Proline content

Free proline content was determined according to the method of Bates et al. ([1973]). Callus was homogenised (1:5, w/v) in 0.05 M ice-cold potassium phosphate buffer (pH 7.0) containing 1 M NaCl, 1 mM EDTA, 1% (w/v) polyvinylpyrrolidone. Then 0.8 ml of 3% sulphosalicylic acid was added to 0.2 ml of the homogenate. The tubes were placed at 4 °C for 10 min and then 1 ml of glacial acetic acid and 1 ml of acid ninhydrin were added. After boiling in a water bath at 100 °C for 60 min, the reaction was stopped by cooling the tubes in ice for 5 min. The chromophore that formed was extracted with 3 ml of toluene by vigorous shaking, and the tubes were placed in the dark for 50 min. Absorbance of the resulting organic layer was measured at 520 nm. The concentration of proline, expressed in nmol g^{-1} FW, was estimated by referring to a standard curve for L-proline.

Statistical analysis

Statistical analyses were performed using the STATISTICA 7.1 programme. To determine individual treatment effects two-way ANOVA at $P \leq 0.05$, followed by Duncans multiple range test was performed .

Results

Two types of media supplemented with kinetin and 2,4-D were used to induce callus formation from the hypocotyls of *M. crystallinum* seedlings: CIM-without NaCl, and CIM-NaCl-containing salt. After 4 weeks of culture, part of the callus tissue was transferred from CIM and CIM-NaCl onto CIM and CIM-NaCl with diethyldithiocarbamate (DDTC) inhibiting SOD or aminotriazole (AT) inhibiting CAT. The following groups of calli cultured on different media were tested: CIM, CIM DDTC, CIM AT and CIM-NaCl, CIM-NaCl DDTC, and CIM-NaCl AT calli. The differences between calli cultured on a medium containing salt and on a medium without salt were found at the morphological and biochemical level (Figs. 1 and 2). CIM callus was non-rhizogenic and exhibited a high amount of betacyanins. The addition of SOD and CAT inhibitors led to a significant decrease in betacyanin content in the CIM DDTC callus as well as in the CIM AT callus. Both calli were friable and had a high rate of growth (Fig. 1A). CIM-NaCl callus possessed rhizogenic potential and, contrary to CIM callus, it exhibited

Fig. 1 Morphology of
M. crystallinum callus (**A**) and
betacyanin content (**B**) in cells
cultured on CIM, CIM-NaCl
and on media supplemented
with AT or DDTC. The same
letters above the *bars* indicate
no statistical difference at
$P \leq 0.05$
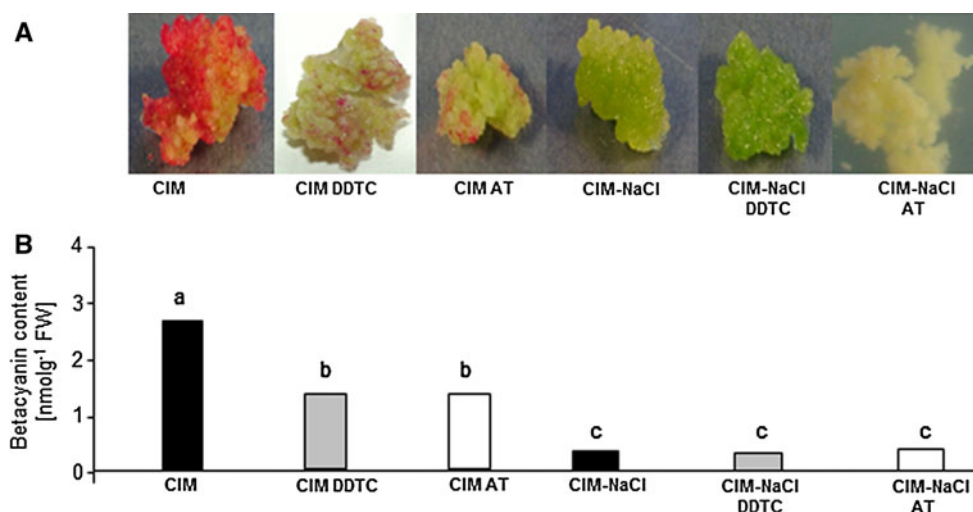
the vestigial quantities of betacyanins (Fig. 1B). As a result of supplementing the growth medium with antioxidant enzyme inhibitors, the CIM-NaCl callus lost its ability to regenerate roots, but the level of betacyanin was not changed. The CIM-NaCl DDTC callus developed fast growing, friable tissue, while callus cultured on the medium with aminotriazole (CIM-NaCl AT) grew very slowly and became necrotic (Fig. 1A). The endogenous level of H_2O_2 in callus tissue cultured on CIM was about five-fold higher than in callus cultured on CIM-NaCl (Fig. 2A). The addition of antioxidant enzyme inhibitors to CIM led to a decrease in H_2O_2 content in CIM DDTC and CIM AT (Fig. 2A). In contrast, the addition of inhibitors to the CIM-NaCl medium resulted in an increase in the H_2O_2 concentration in callus tissue cultured on the CIM-NaCl DDTC medium as well as in callus cultured on the CIM-NaCl AT medium (Fig. 2A). Total SOD activity was lower in callus cultured on the CIM medium as compared to the activity found in callus cultured on the CIM-NaCl medium (Table 1). The addition of DDTC led to a significant decrease in the activities of the CuZn-SOD and MnSOD isoforms, thus lowering total SOD activity in both CIM and CIM-NaCl calii. Aminotriazole inhibition of catalase also influenced the total activity of SOD due to a significant decrease in MnSOD activity (Table 1). The results of CAT activity measurements indicated that callus tissue grown on the medium without NaCl (CIM) exhibited lower activity of CAT as compared to callus cultured on the medium with NaCl (CIM-NaCl) (Fig. 2B). The addition of DDTC did not influence the activity of CAT in either callus cultured on the CIM or the CIM-NaCl, however, AT led to a significant decrease in activity of catalase in CIM AT and CIM-NaCl AT calli (Fig. 2B).

The activity pattern of POD and APX was very similar in relation to the type of medium used for callus production (Fig. 2C and D). The CIM callus exhibited a low level of POD and APX activities when compared to the CIM-NaCl

callus. The addition of DDTC to the CIM medium did not change the activity of either guaiacol or ascorbate peroxidases, but the addition of AT led to a significant increase in POD and APX activity. A significant decrease in the activity of both POD and APX was noted after the addition of antioxidant enzyme inhibitors to the CIM-NaCl medium (Fig. 2C and D).

The level of proline was found to be much lower in the CIM callus than in the CIM NaCl callus tissue (Table 2). The SOD and CAT inhibitors did not influence the level of proline in callus cultured on the medium without salt (CIM DDTC and CIM AT), but the inhibitors did cause a significant change of the proline content in callus cultured on CIM-NaCl. DDTC led to a strong increase in the concentration of proline in the CIM-NaCl DDTC callus, whereas the addition of AT led to a significant decrease in the proline content in the CIM-NaCl AT callus tissue (Table 2).

Discussion

The results presented here revealed that rhizogenesis in the callus cells of *M. crystallinum* was dependent on NaCl addition to the growth medium. For halophytic plants, the role of salt in improving the regeneration rate has already been described (Shi and Ping 2006). It is also well known that different environmental stress factors, including salt stress, cause ROS overproduction and alter the redox potential of the cell. These phenomena can be considered as the primary intracellular events which regulate the function of important cellular components, thus linking external stimuli with signal transduction in stress responses (Adler et al. 1999). Thus, the ROS level and cellular redox potential might lead to the induction of genes responsible for regeneration processes in callus of *M. crystallinum* cultured on a medium supplemented with salt (CIM-NaCl).
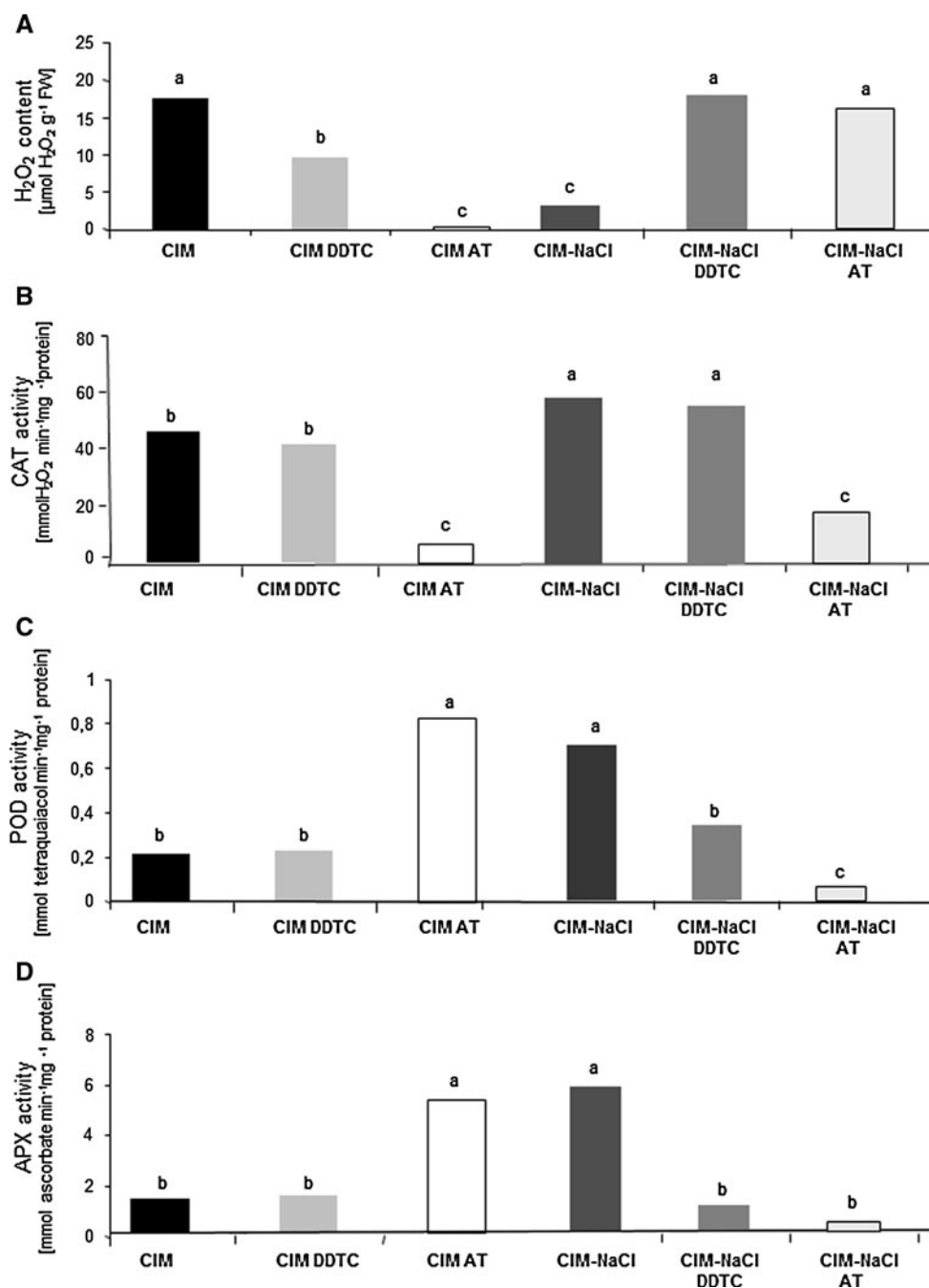
Callus grown on CIM-NaCl exhibited a low level of endogenous H$_2$O$_2$ (Fig. 2A), which was accompanied by root formation, while callus cultured on CIM, without NaCl, was non-rhizogenic and possessed a high level of H$_2$O$_2$ (Fig. 2A). This suggests that the H$_2$O$_2$ metabolism (generation and scavenging) varies between the CIM-NaCl and CIM calli. Indeed, it was found that the pattern of activities of SOD, CAT, APX and POD differed significantly between CIM and CIM-NaCl calli. The total activity of SOD was higher in CIM-NaCl callus than in CIM callus due to stronger induction of CuZnSOD and MnSOD

(Table 1). These isoforms are localized in cytosol and mitochondria respectively, in *M. crystallinum* leaf cells (Miszalski et al. 1998). Moreover, it was recently found that the induction of alternative oxidase activity intensified rooting in the olive tree (Macedo et al. 2009). Therefore, the results of our study concerning SOD activity might support findings on the central role of mitochondria in homeostasis and cell fate determination under stress (Amirsadeghi et al. 2007). Hydrogen peroxide scavenging enzymes exhibited higher activity in the CIM-NaCl callus than in the CIM callus. Similarly, an increase in catalase

Table 1 Activity of superoxide dismutase isoforms (arbitrary units) in the crude extract of *M. crystallinum* callus cultured on CIM and CIM-NaCl as well as on media supplemented with AT or DDTC

SOD form	Medium					
	CIM	CIM DDTC	CIM AT	CIM-NaCl	CIM-NaCl DDTC	CIM-NaCl AT
MnSOD	861b	462c	396c	1651a	627b	663b
FeSOD	538a	474a	441a	433a	367a	451a
Cu/ZnSOD	623b	111c	521b	1967a	832b	1726a
All forms (total activity)	2022c	1047d	1358d	4051a	1817c	2840b

Values represent the activity of SOD forms calculated on the basis of the densitometric analysis of activity stained gels (see "Materials and methods"). Mean values in rows were statistically compared: The same letters indicate no statistical difference at $P \leq 0.05$ (Duncan's test)

Table 2 Proline content (nmolg^{-1}FW) in extract of *M. crystallinum* callus cultured on CIM and CIM-NaCl as well as on media supplemented with AT or DDTC

| Medium					
CIM	CIM DDTC	CIM AT	CIM-NaCl	CIM-NaCl DDTC	CIM-NaCl AT
5.1d	2.9d	4.1d	67.7b	193a	13.9c

Values in row were statistically compared: The same letters above the bars indicate no statistical difference at $P \leq 0.05$ (Duncan's test)

activity in salt-stressed callus tissues was found previously in halophyte *Nitraria tangutorum* Bobr. (Yang et al. 2010) and in sugar cane cells (Patade et al. 2011).

Callus cultured on CIM medium, without NaCl, was characterized by a high amount of betacyanins (Fig. 1). According to previous data betacyanins themselves possess antioxidant properties (Butera et al. 2002; Cai et al. 2003).

The production of betacyanins in *Sueda salsa* suggests that the betacyanin pigment may function as a ROS scavenger, thus limiting the oxidative stress caused by different environmental stress factors, especially light quantity and quality (Wang et al. 2007; Zhao et al. 2010). Moreover, tyrosinase, which is the main enzyme in betacyanin biosynthesis, was found to be able to utilize the superoxide radical to produce melanin in human skin (Perluigi et al. 2003). Thus, it might be assumed, that it may have a similar role in plant tissues. Addition of DDTC or AT to the CIM medium led to a decrease in the content of betacyanins and H_2O_2. It must be mentioned that DDTC as a cooper chelating agent could also have a direct effect on tyrosinase activity (Steiner et al. 1999). The hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and the further oxidation of DOPA, both catalyzed by tyrosinase, are considered to be the first steps in the biosynthesis of betalamic acid and betacyanins (Gandía-Herrero et al. 2005). Therefore, DDTC could affect betacyanin synthesis directly via the inhibition of tyrosinase as well as indirectly via inhibition of SOD, thus leading to the low level of H_2O_2. Catalase deactivation by AT also led to a decrease in the betacyanin content. This phenomenon was accompanied by an increase in the activities of POD and APX. They can function as a substitute for CAT in H_2O_2 removal (Fig. 3). Considering the facts stated above, it can be concluded that a high betacyanin content in CIM callus might be linked to the activity of tyrosinase, which uses the superoxide radical for the production of these pigments, or with a high level of H_2O_2 produced by other sources than SOD.

Measurements of proline concentration (Table 2) confirmed the results of previous studies (Thomas et al. 1992; Kuźniak et al. 2011), suggesting that salinity stress corresponded with a high level of proline in the tissue of *M. crystallinum* L. *M. crystallinum* callus grown in the presence of NaCl also responded to salt treatment by way of high proline accumulation. Proline, induced by stress

Fig. 3 Scheme of the possible link between H_2O_2 content, proline level, betacyanins concentration, activity of some antioxidant enzymes and rhizogenesis potential exhibited by callus cells of *M. crystallinum* cultured on CIM or CIM-NaCl medium
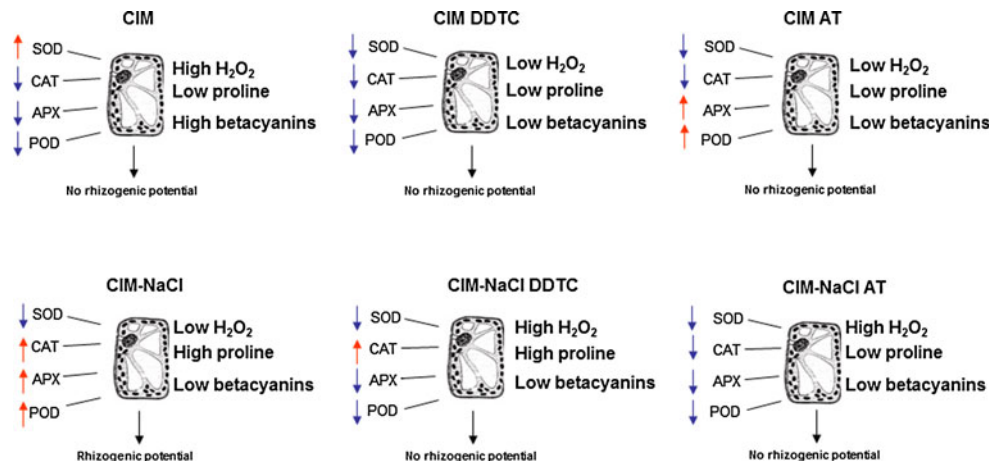
conditions (Kuznetsov and Shevyakova 1999), in addition to its function in the plant cell's response to environmental stresses, plays also an important role in regulating cell morphology and differentiation as well as developmental transitions (Hare et al. 2001). Recently, it was demonstrated that proline is involved in protection against oxidative stress induced by the treatment of salvia leaves with the herbicide paraquat, an elicitor of the superoxide radical production (Radyukina et al. 2008). The experimental system implemented in this research makes it possible to draw the conclusion that a decrease in the total activity of SOD is accompanied by an increase in the proline content (Table 1 and Fig. 3), which suggests that proline, in place of SOD, may function as a scavenger of the superoxide radical, The highest content of proline in CIM-NaCl DDTC callus might support this suggestion. A similar negative correlation was found between proline content and SOD activity in the halophyte *Thellungiella halophila*, and it was suggested that a high level of proline in this plant may compensate low SOD activity and contribute to salt resistance (Kartashov et al. 2008). In our studies the addition of DDTC inhibiting SOD (Table 1) or AT inhibiting CAT (Fig. 2) to the CIM-NaCl medium did not correspond with a decrease in the H_2O_2 content in the callus cells. On the contrary, a much higher concentration of H_2O_2 and a loss of rhizogenic ability in this callus were observed (Fig. 3). The addition of DDTC to the CIM-NaCl medium was accompanied by an increase in the level of H_2O_2, probably due to the decrease in the activity of H_2O_2 scavenging enzymes, such as POD and APX (Fig. 3). The inhibition of CAT in the CIM-NaCl AT callus tissue also led to the loss of rhizogenic potential, however, in this case a high level of H_2O_2 led to the induction of necrosis. All data mentioned above allows us to conclude that the ability of CIM-NaCl callus to regenerate roots might correlate with a high proline content synthesized in response to a high concentration of the superoxide radical and a low level of hydrogen peroxide regulated by antioxidant enzymes, including: CAT, APX and POD (Fig. 3).

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