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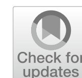
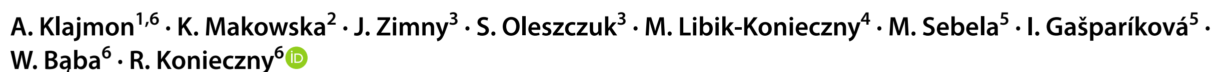


Gum Arabic influences the activity of antioxidant enzymes during androgenesis in barley anthers



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Abstract

This study investigated the antioxidant activity of gum Arabic (GA) during androgenesis in barley anthers. After stress pretreatment the anthers were cultured in the presence of 10 mg L^{-1} GA (Gm) or on control medium (Cm) and compared with respect to activity of selected antioxidant and respiratory enzymes and endogenous hydrogen peroxide (H_2O_2) content. The anthers from Cm and Gm differed in the strategy against H_2O_2 overproduction, in that the total peroxidase (POX) activity and the number of POX isoforms were significantly higher in Gm- than in Cm-cultured material. High POX activity on Gm paralleled with H_2O_2 decrease, suggesting the utilization of this chemical for the POX-mediated cell wall formation and reconstruction during growth of multicellular structures. The total superoxide dismutase (SOD) activity on Cm and Gm were at similar level for most of the culture period but the activity of MnSOD was dozen times higher on Gm and this coincided with high activity of fumarase and cytochrome *c* oxidase. It indicates close interplay between efficient antioxidative protection and high metabolic rate accompanying efficient androgenesis. Mass spectrometry analysis confirmed the presence of POX and other antioxidative and defense enzymes in protein fraction of GA used in the experiments, however as revealed by 2,2-diphenyl-1-picrylhydrazyl assay, the Gm and Cm displayed similar total antioxidant capacity. Thus, the effect of GA on androgenic cultures of barley can be linked to its influence on the activity of anther antioxidant system, rather than its native antioxidative properties.

Key message

This study shows a high activity of antioxidant enzymes in barley anthers exposed to gum Arabic. Effective ROS scavenging coincides with high respiratory activity of anthers and effective androgenesis.

Keywords *Hordeum vulgare* · Metabolism · Microspore embryogenesis · Oxidative stress · Total antioxidant capacity

Abbreviations

AS	Anthers after stress treatment
BS	Anthers before stress treatment
CAT	Catalase
Cm	Control medium
COX	Cytochrome *c* oxidase
FUM	Fumarase
GA	Gum Arabic

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Gm Cm medium supplemented with 10 mg L^{-1} gum Arabic
POX Peroxidase
SOD Superoxide dismutase

Introduction

Androgenesis is a development of plant from haploid microspore (Maraschin et al. 2005). Aside importance in plant breeding programs e.g., the production of homozygous double haploid plants via androgenesis, it offers a unique system for studying the mechanisms underlying totipotency of plant cell.

Plant regeneration via androgenesis can be obtained in in vitro culture of anthers or microspores by the application of various inducing factors, among which the stress pre-treatment of explants is considered as the most effective (Tajedini et al. 2022). It is believed that reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{-\cdot}$) produced during stress pre-treatment, act as signaling molecules involved in the switch of microspores from their natural development into pollen grains towards an embryogenic pathway (Rodriguez-Serrano et al. 2009; Żur et al. 2019). ROS are also involved in the modification and de novo formation of cell wall, and these processes are of crucial importance for proper divisions of microspores and further growth and differentiation of multicellular structures (MCSs) (Uvačkova et al. 2012). However, ROS are also mediators of programmed cell death (PCD) which is one of the main reasons for decrease in the efficiency of androgenic regeneration (Rodriguez-Serrano et al. 2009). When they are overproduced, ROS can cause direct damage to the basic building blocks of the cell including DNA, protein and lipids (Shields et al. 2021). This dual function of ROS requires an efficient control over their production and scavenging throughout androgenic development. This can be achieved among others, by the adjustment of cellular antioxidant system activity, e.g., by the changes in the expression and/or activity of antioxidative enzymes such as superoxide dismutases (SOD; EC 1.15.1.1), peroxidases (POX; EC1.11.1.7) and catalases (CAT; EC 1.11.1.6) (Mittler et al. 2004, 2011).

In barley (*Hordeum vulgare* L.), due to the agronomic importance of the species, numerous protocols for plant regeneration via androgenesis have been described (Wrobel et al. 2011; Castillo et al. 2014; Makowska et al. 2015; Patial et al. 2022). Despite of this, there is still the need to optimize the culture conditions for higher efficiency of regeneration, and especially to minimize the production of albino plants (Makowska et al. 2014). In previous studies, we have found that addition of gum Arabic (GA) to the culture medium positively affects androgenesis from barley anthers (Makowska et al. 2017). Similar, beneficial effect of GA on regeneration in vitro has been reported only in a few species, e.g., wheat (Letarte et al. 2006) and *Eucalyptus globulus* (Corredoira et al. 2015), but the mechanisms underlying this phenomenon remains unclear.

GA is a sticky liquid that exudes from the stems of *Acacia senegal* and *Acacia seyal* in response to environmental stresses including heat, drought or wounding (Mahendran et al. 2008). As revealed by chemical studies, GA is a composite of polysaccharides and glycoproteins and in addition, high amount of Ca^{2+}, Mg^{2+}, germin-like proteins and several enzymes, such as laccases and POX were detected in GA (Islam et al. 1997; Mariod 2018; Musa et al. 2018). From studies on animal and human cells it has been evident that the physiological effects of GA application are cell-specific and can include among others the reduction of ROS level, decrease of oxidative stress, activation of cellular antioxidant enzymes, and increase of total antioxidant capacity of cells (e.g., Ahmed et al. 2016; Ayaz et al. 2017; Kaddam et al. 2017; Ali et al. 2020). In barley androgenesis, addition of GA to the medium stimulated the process by reduction of microspores mortality, shortening the time required for the first sporophytic microspore division and by increasing the production of MCSs from which embryogenic callus developed (Makowska et al. 2017). From studies on other plant species, it has been known, that all these processes are either directly, like PCD or indirectly like, MCSs development controlled by ROS (Rodriguez-Serano et al. 2009; Huang et al. 2019) generated at the early stages of androgenic induction.

The main goal of our study was to analyse the antioxidant activity of GA during androgenesis in barley anthers. For this purpose, we evaluated the effect of GA on endogenous level of H_2O_2 and activity of antioxidant enzymes, SOD, CAT and POX in the anthers. Next, we verified as to whether GA-increased activity of antioxidant enzymes coincides to high respiratory intensity of anthers—a phenomenon which was shown to accompany the onset and progression of efficient regeneration in vitro (Konieczny et al. 2014). For this purpose, we measured the activity of key respiratory enzymes, fumarase (FUM) and cytochrome *c* oxidase (COX) in the anthers maintained on Cm and Gm. We also tested whether the antioxidant effect of GA on barley anthers can be attributed to any GA-specific antioxidant and whether this effect is direct or indirect, i.e., through modulation of the anther antioxidant system. To answer these questions, we analysed the composition of protein fraction of GA and compared the total antioxidant activity of Cm and Gm. The results we obtained brought about new insight into poorly understood mechanisms of GA's action on plant cells.

Materials and methods

Plant material

The studies were carried out on spring barley (*Hordeum vulgare* L.) NAD19 line kindly provided by the breeding company Poznańska Hodowla Roślin LTD-Negradowice, Poland. Conditions for donor plant growth were described previously (Makowska et al. 2017). The seeds were sown on 10 cm Petri dishes lined with moistened filter paper and kept in the dark for 5 days at 4 °C followed by 7 days at 16 °C. After reaching about 18 cm in height, the plants were placed in pots containing a mixture of soil and sand (3:1 v/v) in the phytotron at 16/12 °C (day /night) and 240 µM m^{-2} s^{-1} light provided by sodium lamps (Makowska et al. 2017).

In vitro cultures

The anthers containing microspores at the uninucleate stage were isolated from spikes and subjected to surface sterilization in 70% (v/v) ethanol for 1 min, followed by 10% (v/v) sodium hypochlorite for 20 min. Then, the anthers were rinsed five times with sterile distilled water. The sterilized material was placed on Petri dishes (diameter 10 cm) filled with 10 mL of pretreatment solution containing 62 g L^{-1} of mannitol and 2.5 mg L^{-1} of copper (II) sulfate ($CuSO_4$) for 5 days at 4 °C. To study the effect of gum Arabic (GA) on the activity of respiratory and antioxidant enzymes and endogenous H_2O_2 content the anthers were cultured on two different agar-solidified media: control medium (referred here as Cm) consisting of N6L macro- and microelements (Chu 1978) and growth regulators according to Makowska et al. (2015) and gum GA-containing medium (referred here as Gm) with the composition as mentioned above but with the addition of 10 mg L^{-1} of gum Arabic (Fagron, Poland). The effect of GA was compared with respect the activity of the following enzymes: CAT, POX, SOD, FUM and COX. All media were solidified with 0.8% (w/v) agar (Difco, USA), adjusted to pH 5.7 with 1N NaOH and/or 1N HCl and autoclaved for 20 min under 1 MPa. The in vitro cultures were carried out on Petri dishes (100 anthers per 10 cm dish) under the conditions described elsewhere (Makowska et al. 2017).

Material for biochemical studies

The material used in the biochemical studies were the anthers, protein fractions of GA and culture media i.e., Cm and Gm. The anthers before and after stress pretreatment (BS and AS, respectively) as well as the anthers after 3 and 7 days of culture on Cm (A3Cm and A7Cm, respectively) and Gm (A3Gm and A7Gm, respectively) were used for studies on the effect of GA on the antioxidant and respiratory enzyme activities and endogenous H_2O_2 content during androgenesis. The protein fractions of GA were analysed for the presence of POX and other enzymes related to metabolism of H_2O_2, whilst the Cm and Gm were compared with respect to total antioxidant capacity.

Biochemical studies on anthers

Protein isolation

To isolate fractions of soluble proteins, 1 g of fresh material was homogenized at 4 ºC with a mortar in 1 cm^3 of extraction 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). The homogenate was centrifuged for 5 min at 5000×*g*, then the supernatant was collected. The protein concentration in soluble fraction of proteins isolated from the anthers was determined according to Bradford (1976) using the Bio-Rad Protein Assay Kit with BSA as standard. Protein fractions were stored at − 80 ºC until further use.

Visualization and determination of SOD and POX activities

To visualize the activity of SOD, the fractions of soluble proteins isolated from anthers were separated using native PAGE at 4 ºC and 180 V in the Laemmli buffer system (Laemmli 1970) without sodium dodecyl sulfate (SDS). Each lane was loaded with 15 µg (for SOD) and 10 µg (for POX) of total protein content in the extracts.

SOD activity bands were visualized according to the procedure described by Beauchamp and Fridovich (1971). The gels were incubated in staining solution (potassium phosphate buffer, pH 7.8, containing 0.0068 g L^{-1} KH_2PO_4, 0.0175 g L^{-1} Na_2HPO_4, 0.372 g L^{-1} EDTA, 31% (v/v) TEMED, 7.5 mg L^{-1} riboflavin and 0.2 g L^{-1} NBT for 30 min in the dark at room temperature and then exposed to white light until SOD activity bands became visible. For particular isoforms identification 5 mM H_2O_2 was added to the staining buffer in order to inhibit Cu/ZnSOD and FeSOD. The inhibition of Cu/ZnSOD was achieved by incubating the gels in a buffer containing 3 mM KCN.

POX activity bands were visualized using the activity-staining procedure described by Graham et al. (1964). The gels were incubated in staining solution (potassium phosphate buffer, pH = 5, containing 0.0068 g L^{-1} KH_2PO_4, 0.0175 g L^{-1} Na_2HPO_4, 0.4285 g L^{-1} DAB in DMSO) for 20 min. Then, 25 mL of an aqueous solution of 30% hydrogen peroxide was added to the staining buffer. Directly after the appearance of the bands, gels were transferred to water and placed at 4ºC. The gels were kept at 4 °C until densitometric analysis.

The activity of SOD and POX was determined using the densitometric method. The gels were scanned using Epson Perfection V600 Photo scanner, and then analyzed in the ImageJ program (NIH ImageJ, National Institutes of Health, Bethesda, USA, http://rsb.info.nih.gov/ij/index.html). Activity of enzymes was expressed in arbitrary units corresponding to the area under the densitometric curve.

CAT activity

CAT activity in the anther's extract of soluble proteins was determined spectrophotometrically using the method described by Aebi (1984). Reaction was run in 1 mL cuvette containing 10 µg of the protein extract in phosphate buffer (0.0068 g L^{-1} KH_2PO_4, 0.0175 g L^{-1} Na_2HPO_4 pH 7.0). The absorbance was measured at 240 nm using a UV lamp for 2 min. The results were calculated using an extinction coefficient for H_2O_2 of 43 mM^{-1} cm^{-1}. CAT activity was expressed as the amount of the decomposed substrate (H_2O_2) by 1 mg of protein per minute [µM min^{-1} mg^{-1}].

Endogenous content of H_2O_2

The endogenous concentration of H_2O_2 in the anthers was determined according to the modified procedure of Brennan and Frenkel (1977). The extract was prepared by homogenization of 1 g of plant material in a mortar in 2 mL of cold acetone. After centrifugation for 5 min at 10,000×*g* the pellet was discarded and 0.5 mL of supernatant was used for further analysis.

A 100 µL of the titanium chloride ($TiCl_4$) solution (0.5 mL of a 90% aqueous solution of $TiCl_4$, 2.5 mL 30% HCl) and 200 µL of 1 M NH_4Cl were added to the supernatant and shaken in the vortex for several minutes. The resulting precipitate, i.e., the titanium- peroxide complex, was centrifuged for 5 min at 10,000×*g*. The purified solid was poured into 2 mL 2N H_2SO_4 and agitated until complete dissolution and a clear solution was obtained. Absorbance was measured at a wavelength of 415 nm against water blank. The final concentration of peroxide in the extract was determined by comparing absorbance against the standard curve representing the titanium-H_2O_2 complex over a range from 0 to 0.2 µM. The measurements were normalized to a fresh weight of tissue.

FUM activity

To analyse FUM activity, the anthers (0.3 g) were homogenized on ice in 0.6 mL 0.1 M HEPES–KOH, pH 8.0, containing 2 mM DTT, 2 mM $MnCl_2$, and 1% (w/v) PVP-40. Nonsoluble material was removed by centrifugation for 2 min at 12,000×*g* and the supernatant was immediately used for measurements of enzymatic activity. Spectrophotometric determination of FUM activity was performed according to Miszalski et al. (2001) at 240 nm. The reaction was performed in 50 mM Tricine-KOH, pH 8.0, containing 0.02% (w/v) TritonX-114 and 60 mM L-malate. FUM activity was calculated using extinction coefficient for fumarate of 2.44 mM^{-1} cm^{-1} and expressed as the amount of fumarate produced by 1 mg of protein per minute [nM min^{-1} mg^{-1}].

COX activity

Activity of COX was analysed in the extracts of anthers prepared from 0.3 g of fresh tissue homogenised on ice in 0.6 mL of 150 mM phosphate buffer pH 7.5, containing 0.02% (w/v) TritonX-114. Spectrophotometric determination of COX activity was performed according to Kato et al. (1997) at 550 nm. The reaction was studied in 150 mM phosphate buffer containing 15 µM L^{-1} cytochrome *c* that was reduced before starting the reaction to 90% using *N*-dithionite. The decrease in the absorbance at 550 nm was followed and COX activity was calculated using extinction coefficient for reduced cytochrome *c* of 21 mM^{-1} cm^{-1} and expressed as the amount of reduced cytochrome *c* oxidized by 1 mg of protein per minute [nM min^{-1} mg^{-1}].

Biochemical studies on GA

Protein isolation

To prepare protein extract 10 g of GA powder (Sigma, Germany) was dissolved in 100 mL cold *2-amino-2-*(hydroxymethyl) *propane-1*,*3-diol* in Tris–HCL, pH 7.0, mixed for 3 h on ice bath followed by centrifugation for 12 h (3600 g, 5 °C). The supernatant was precipitated with cold acetone (1:4 v/v) and the resulting pellet was dissolved in 1 mL Tris HCl, pH 7 and spin down. The protein concentration in the supernatant was determined using BCA kit (BCA Protein Assay Kit, Thermo Fisher Scientific, USA) according to manufacturer protocol. Protein fractions from GA were stored at -80 ºC until further use.

Determination of POX activities

The activity of POX in protein fractions of GA was measured spectrophotometrically. The measurements were done according to the method described by Pütter (1974). Reaction was run in 1 mL cuvette filled with 10 µL of extract, 1 M phosphate buffer pH 6.0, 54 µL 0.5% (v/v) H_2O_2 and 107 µL 5% (v/v) guaiacol (Sigma, Germany). The absorbance was measured at 470 nm for 5 min The activity of POX was calculated using an extinction coefficient for tetraguaiacol of 26.6 mM^{-1} cm^{-1}. POX activity was expressed as the

amount of production of tetraguaicol by 1 mg of protein per minute [nM min^{-1} mg^{-1}].

POX identification in GA by mass spectrometry

GA samples were dissolved in deionized water (5%, w/v) and dialyzed against deionized water overnight. Centrifuged aliquots (1 ml) of the dialyzed solutions were separated by gel permeation chromatography on a glass column (1.5 cm i.d. × 30 cm) packed with Sephacryl S-200 HR (GE Healthcare, Uppsala, Sweden). The column was attached to a peristaltic pump PCD1082 (Kouřil, Kyjov, Czech Republic) providing a flow rate of the mobile phase (deionized water) of 1 ml/min. The eluate was monitored by UV absorption at 280 nm using a UV-1 control and optical unit (Pharmacia LKB, Uppsala, Sweden). Protein fractions were collected after a visual inspection of the chromatogram (obtained using a REC-112 recorder by Amersham Pharmacia Biotech, Uppsala Sweden) in 2-ml aliquots. Each aliquot was evaporated in a vacuum centrifuge and the solid residue dissolved in 20 µl of Laemmli sample buffer for SDS-PAGE or by adding 15 µl water, 15 µl 50% (v/v) glycerol and 2 µl ampholytes (Pharmalyte 3–10; GE Healthcare) prior to isoelectric focusing (IEF). The electrophoretic separations in polyacrylamide gels were run according to previous protocols for SDS-PAGE (Laemmli 1970) and native/denaturing IEF (Robertson et al. 1987). Samples of 20 µl were loaded per gel well. The gels were stained by Bio-Safe Coomassie stain (Bio-Rad, Hercules, CA, USA), in the case of IEF after preceding washing steps with trichloroacetic acid solutions and water (Robertson et al. 1987). GA proteins were additionally analyzed after their precipitation from 100 ml of the 5% (w/v) solution by ammonium sulfate (80% of saturation), dissolution in 2 ml of 20 mM potassium phosphate buffer (pH 7.0), dialysis against the buffer and ultrafiltration to a achieve a protein concentration of around 1 mg/ml. The samples were then separated by SDS-PAGE and processed as above.

Protein bands were excised from the gel slabs and processed for in-gel digestions by SOLu trypsin (Merck, Steinheim, Germany) as described (Shevchenko et al. 2006). Peptides from the digests were separated by reversed-phase nanoflow liquid chromatography (nLC) coupled either to MALDI or ESI tandem mass spectrometry (MS/MS) for protein identification (Petrovská et al. 2014). The MGF-formatted files from the MS/MS analyses were also searched using PEAKS X Studio (Bioinformatic Solutions Inc., Waterloo, ON, Canada) against a custom protein database for the genus *Acacia* (102514 sequences, originating particularly from *A. pycnantha* and *A. crassicarpa*), which was downloaded from the NCBI Protein database in December 2022. The adjusted search parameters included MS and MS/MS mass tolerances of 50 ppm and 0.5 Da, respectively, trypsin as a protease (semispecific; up to 2 missed cleavages), and carbamidomethylation of Cys as a fixed modification. The choice of variable modifications included oxidation of Met, deamidation of Asn and Gln, and acetylation of the N-terminus.

Total antioxidant capacity of media

Total antioxidant capacity of Cm and Gm was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma, Germany) according to Brand-Williams et al (1995) with modifications adapting the procedure to measurement with a microtitration plate reader (Płażek et al. 2011). A solution of 0.5 mM of DPPH in methanol was used. Before measurement the media and GA extract were allowed to react with DPPH solution for 20 min in the dark, at 25 °C. The absorbance decreasing was recorded by plate reader Model 680 (Bio-Rad Laboratories, USA) at 515 nm. The antioxidant capacity of the samples was calculated using a standard curve drawn up for solutions of Trolox (Sigma, Germany) and expressed as µM dm^{-3} of Trolox equivalents.

Statistical analysis

For each experiment, the means of 3–5 replicates were calculated. The experiments were repeated three times. Statistically significant differences between means ($p \leq 0.05$) were determined by a one-way ANOVA followed by Duncan's multiple range test using Statistica for Windows ver. 8.0 (StatSoft, Inc. Tulsa, OK, USA).

Results

GA added to the medium affects the activity of antioxidant enzymes and endogenous content of H_2O_2 in barley anthers

SOD activity

Three bands corresponding with the activity of different SOD isoforms were visualized on gels after electrophoretic separation of proteins isolated from anthers (Fig. 1, Table 1). Based on selective inhibition of particular SOD isoforms (Miszalski et al. 1998) the upper band was identified as MnSOD whilst two lower bands as Cu/ZnSOD-1 and Cu/ZnSOD-2. The activities of Cu/ZnSOD were detected in all material studied, whilst the activity of MnSOD was not confirmed in BS. Among evaluated samples, the highest total SOD activity was found in AS and BS. At the third day of culture on Cm and Gm, total SOD activity dropped and the decrease was more pronounced in the anthers maintained

on Cm (Table 1). The total activity of SOD increased reaching the similar level in the anthers cultured on Cm and Gm by day 7. The contribution of individual SOD isoforms to total SOD activity changed according to the time of culture and type of the media. Throughout the culture period the activity of MnSOD was several times higher in the anthers maintained on Cm when compared to Gm but the time of culture had no effect on the activity of this enzyme. In case of Cu/ZnSOD, the highest activities of its two isoforms were noted in BS and AS followed by conspicuous decrease after explantation onto Cm and Gm. The decrease in the activity of Cu/ZnSOD-1, however, was more pronounced than this observed for Cu/ZnSOD-2. With continued culture the activity of Cu/ZnSOD-1 doubled by day 7 on Cm and Gm, whilst the activity of Cu/ZnSOD-2 either did not change or remained at the same level as determined for day 3 or doubled when maintained on Gm and Cm, respectively.

POX activity

The highest total POX activity and the highest number of POX isoforms were observed in BS and AS (Fig. 2, Table 2).

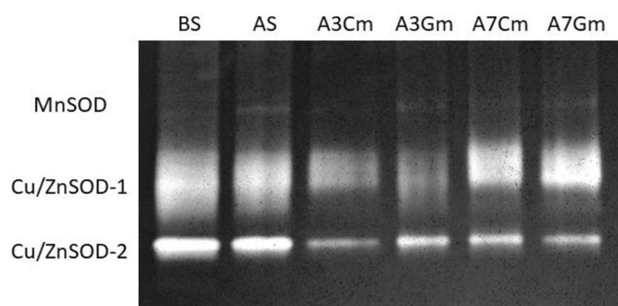

Fig. 1 The patterns of SOD isoforms in barley anthers before and after stress treatments (BS and AS, respectively) as well as in the anthers after 3 and 7 days of culture on Cm (A3Cm and A7Cm, respectively) and Gm (A3Gm and A7Gm, respectively). Each well was loaded with 15 mg of protein

Initiation of in vitro culture brought about conspicuous decrease in total POX activity but the drop was much more pronounced on Cm than Gm. In all samples maintained in vitro the POX-1 was the main isoform contributed to total activity of the enzyme. Its activity, however, was several folds lower in the material maintained on Cm than Gm (Table 2). Among the six POX isoforms which were detected in BS and AS only the very low activity of few isoenzymes was confirmed in the material maintained on Cm. These were POX-1 and POX-2 which were detected at day 3 of culture and POX-1 which activity was found at day 7. The time of culture on Cm had no effect on the total activity of POX which was kept at the similar level at day 3 and 7 of culture and was the lowest among the samples studied. In opposite to the anthers cultured on Cm, the number of active POX isoforms increased with time on Gm, so that after 7 days of inoculation all isoforms identified in BS and AS were detected in the material exposed to GA (Fig. 2). The increase in the number of active POX isoforms on Gm was accompanied by an increase in total activity of the enzyme which at the 7 day of culture was about 1.5-fold higher than this found at day 3 on Gm and about 40-times higher when compared to the anthers maintained on Cm.

CAT activity

In the anthers before and after stress treatment, the activity of CAT was significantly higher than in the explants maintained in vitro (Fig. 3). At the 3rd day of culture on Cm and Gm the activity of this enzyme was at the similar level and about twice as low as before culture initiation. Further culture on Cm and Gm did not affect the activity of CAT which at the 7 day of culture was comparable to those found at day 3.

Table 1 Activity of SOD isoforms (arbitrary units) in the crude extract from barley anthers before and after stress treatments (BS and AS, respectively) as well as from the anthers after 3 and 7 days of culture on Cm (A3Cm and A7Cm, respectively) and Gm (A3Gm and A7Gm, respectively)

	Plant material					
	BS	AS	A3Cm	A3Gm	A7Cm	A7Gm
MnSOD	0	108^{c}	6^{a}	72^{b}	4^{a}	62^{b}
Cu/ZnSOD- 1	624^{c}	522^{c}	171^{a}	161^{a}	372^{b}	379^{b}
Cu/ZnSOD- 2	838^{c}	776^{c}	227^{a}	592^{b}	496^{b}	484^{b}
Total	1462^{c}	1406^{c}	402^{a}	825^{b}	872^{b}	925^{b}

Values represent the activity of SOD isoforms calculated on the data obtained from the densitometric analysis of activity-stained gels (see "Materials and methods" section). Mean values in rows were statistically compared. The same letters indicate no statistical difference at $p \leq 0.05$ according to Duncan's multiple test

Endogenous concentration of H_2O_2

The endogenous content of H_2O_2 significantly increased in the anthers during the incubation in the pretreatment solution, followed by a sharp decline within the 3 day-long culture period on both Cm and Gm (Fig. 4). At the 3 day of culture the anthers plated on Cm contained about 2-times more H_2O_2 compared with those from Gm, in which, the endogenous content of H_2O_2 was at the same level as in BS. With continued culture on Cm the content of H_2O_2 remained constant, whilst it sharply decreased in the anthers maintained on Gm. Finally, at 7 day on Gm the H_2O_2 concentration in the explants was ca sevenfold lower than in the explants from Cm and about 3.5-fold lower than in BS.

Cm and Gm display similar total antioxidant capacity

To evaluate whether the observed differences in antioxidant enzyme activity and H_2O_2 content between the anthers maintained on Cm and Gm are related to different antioxidant properties of these media, we determined the total antioxidant capacity of Cm and Gm by DPPH method. The results revealed that total antioxidant capacity of these two-culture media expressed in microgram Trolox equivalents was similar (0.072 ± 0.0012 and 0.075 ± 0.0005 for Cm and Gm, respectively) indicating no effect of GA on free radical-scavenging activity of the medium. As revealed by spectrophotometric analysis, increasing the amount of GA in the Cm up to 10 g L^{-1} had no effect on the total activity of the medium, as well (data not shown).

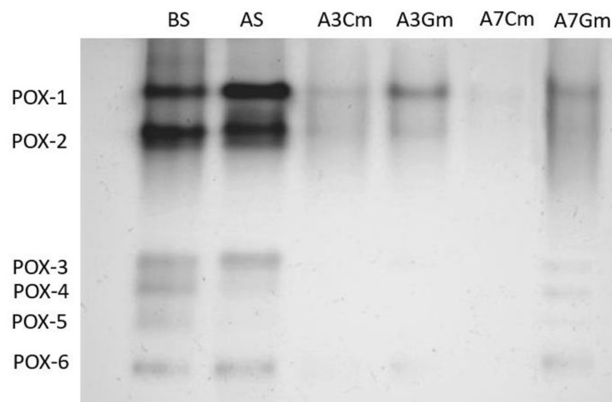

Fig. 2 The patterns of POX isoforms in barley anthers before and after stress treatments (BS and AS, respectively) as well as in the anthers after 3 and 7 days of culture on Cm (A3Cm and A7Cm, respectively) and Gm (A3Gm and A7Gm, respectively). Each well was loaded with 10 μg of protein

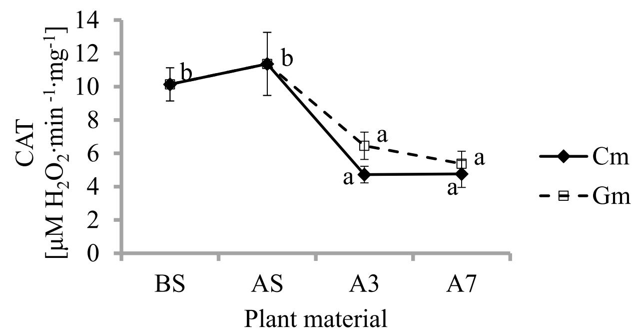

Fig. 3 CAT activity in barley anthers before and after stress treatments (BS and AS, respectively) as well as in the anthers after 3 and 7 days of culture (A3 and A7, respectively). Data are averages ± SD; values in series sharing the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple test.

Table 2 Activity of POX isoforms (arbitrary units) in the crude extract from barley anthers before and after stress treatments (BS and AS, respectively) as well as from the anthers after 3 and 7 days of culture on Cm (A3Cm and A7Cm, respectively) and Gm (A3Gm and A7Gm, respectively)

	Plant material					
	BS	AS	A3Cm	A3Gm	A7Cm	A7Gm
POX-1	91^d	101^d	8^a	69^c	3^a	32^b
POX-2	143^b	150^b	5^a	3^a	0	2^a
POX-3	61^b	97^c	0	0	0	8^a
POX-4	66^c	41^b	0	0	0	12^a
POX-5	16^b	9^a	0	0	0	2^a
POX-6	68^c	63^c	0	0	0	44^b
Total	445^c	461^c	13^a	72^b	3^a	118^c

Values represent the activity of POX isoforms calculated based on the densytometric analysis of activity-stained gels (see "Materials and methods" section). Mean values in rows were statistically compared. The same letters indicate no statistical difference at $p \leq 0.05$ according to Duncan's multiple test

Addition of GA to the medium influences the activity of respiratory enzymes in barley anthers

FUM activity

Among the samples studied, the lowest activity of FUM was determined in the anthers after stress treatment (Fig. 5). During the first 3 days of culture on Cm and Gm the activity of this enzyme increased compared to AS, but the rise was much more pronounced in the material cultured on Gm than Cm. Further culture on Gm resulted in conspicuous about2-fold increase in FUM activity, whilst in the anthers maintained on Cm, the activity of the enzyme remained unchanged.

COX activity

Both stress treatment and time of culture on Cm had no effect on the activity of COX (Fig. 6). In case of explants maintained on Gm, the activity of COX increased with the time reaching the highest values at day 7 of culture. Throughout the culture period the activity of COX in the anthers maintained in the presence of GA was about twice as high as explants cultured on Cm at the respective days.

GA used in the experiment contains POX and other antioxidant and defense proteins

GA proteins precipitated by ammonium sulfate and analyzed by SDS-PAGE provided a separation pattern (Fig. 7), which was dominated by thick bands at 30–35 kDa and 150 kDa. Other protein bands appeared at 13, 16, 22–25 and around 60 kDa. Protein identification performed by nLC-MALDI-MS/MS after in-gel digestion revealed the presence of POX (assigned to gi: 2236253595 from *Acacia crassicarpa* with a theoretical molecular mass of 34 kDa), laccase (gi:2313549411 or gi:2236226242; *A. pycnantha* or *A. crassicarpa*, respectively; 63 kDa), thaumatin-like protein (gi:2313544319; *A. pycnantha*; 24 kDa) and pathogenesis-related protein (gi:2313554112; *A. pycnantha*; 16 kDa). Gel permeation chromatography was involved to separate GA proteins more efficiently. The collected fractions contained a protein material, which could be visualized by staining with Coomassie Brilliant Blue after electrophoretic separations (SDS-PAGE or IEF). Again, the visualized proteins in gels were subjected to a procedure of in-gel tryptic digestion followed by nLC separation of peptides coupled to MALDI or ESI–MS/MS analysis for protein identification. The MS/MS data were searched against *Acacia* sequences downloaded from the NCBI Protein database. Electronic Supplementary Material summarizes the retrieved results when at least two assigned peptides in repeated analyses were necessary to consider the identification valid. Various defense-related proteins were found, such as oxidative enzymes (POX, laccase, aldehyde oxidase, germin-like and berberine bridge

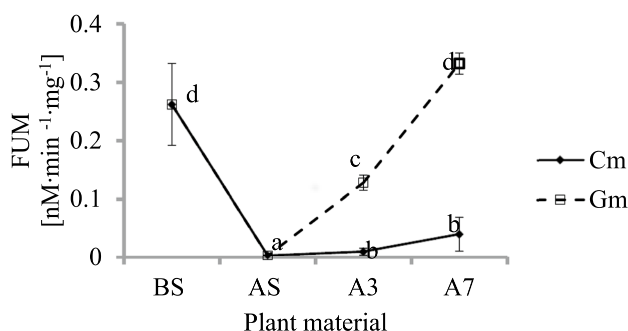

Fig. 5 FUM activity in barley anthers before and after stress treatments (BS and AS, respectively) as well as in the anthers after 3 and 7 days of culture (A3 and A7, respectively). Data are averages ± SD; values in series sharing the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple test

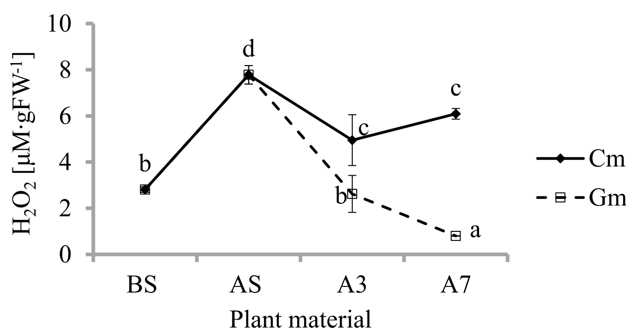

Fig. 4 Endogenous H_2O_2 content in barley anthers before and after stress treatments (BS and AS, respectively) as well as in the anthers after 3 and 7 days of culture (A3 and A7, respectively). Data are averages ± SD; values in series sharing the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple test

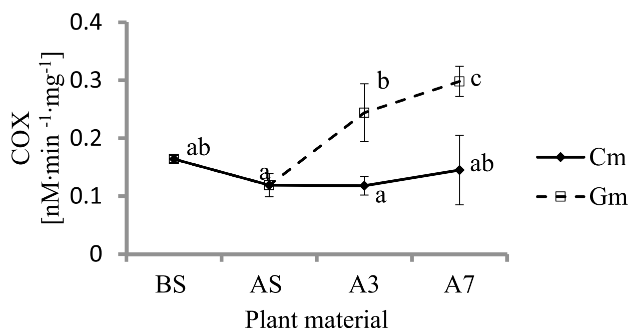

Fig. 6 COX activity in barley anthers before and after stress treatments (BS and AS, respectively) as well as in the anthers after 3 and 7 days of culture (A3 and A7, respectively). Data are averages ± SD; values in series sharing the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple test

enzyme-like proteins), pathogenesis-related (PR) proteins (chitinases, glucan-1,3-β-glucosidases and thaumatin-like proteins) or protease inhibitors. The specific activity of POX in GA was 0.35 $nkat \cdot mg^{-1}$ as determined by the spectrophotometric assay with hydrogen peroxide and guaiacol.

Discussion

This study is a continuation of our previous research in which we described a beneficial effect of gum Arabic (GA) on androgenesis in barley anthers culture (Makowska et al. 2017). Our objective in this paper was to investigate the antioxidative action of GA during early stages of androgenesis.

In order to induce androgenesis, the barley anthers were incubated in a pre-treatment solution (mannitol + $CuSO_4$) at 4 °C for 5 days and this was accompanied by conspicuous accumulation of H_2O_2 in the explant tissues. Generation of ROS, such as H_2O_2, has been suggested as the first, although not the only, prerequisite for initiation of sporophytic development of microspores (Żur et al. 2009). However, prolonged overproduction of ROS can be detrimental to normal cellular function, and in addition, H_2O_2 can trigger programmed cell death (PCD) (Smirnoff and Dominique 2019). In a previous study, we found that addition of GA to induction medium significantly reduced microspore mortality (Makowska et al. 2017), whilst in this research we report that endogenous concentration of H_2O_2 in the anthers after 3 days on Gm was almost half of those determined on Cm. These observations point to protective function of GA against oxidative stress as well as to the link between its antioxidative properties and improvement of androgenetic response. Achieved data also seem to confirm the statement of Rodriguez-Serrano et al. (2012) that scavenging of excess ROS shortly after stress treatment is a key event decreasing microspore death and increasing androgenic response.

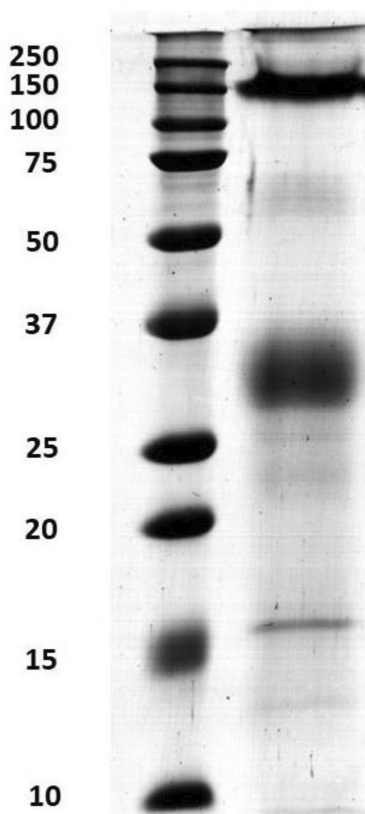

Fig. 7 SDS-PAGE of GA proteins. The sample was obtained by ammonium sulfate precipitation followed by dissolution of the precipitate, dialysis and ultrafiltration. From the left, Precision Plus Protein Standards Kaleidoscope (Bio-Rad) with the indicated molecular mass values (in kDa), GA protein sample (15 µg). The gel was stained with a colloidal Coomassie Brilliant Blue R-250 stain

Antioxidative properties of GA have been ascribed to the presence of oxidising enzymes, especially POX (e.g., EFSA 2017, Mirghani et al. 2018). As revealed by MS/MS analysis performed here, the protein fraction of GA contained different antioxidative enzymes and the activity of GA-specific POXs we confirmed spectrophotometrically. However, according to DPPH assay, the induction medium containing GA did not differ with respect to total antioxidant capacity from control one. In studies on mango, Khaliq et al. (2015; 2016) reported that tolerance of fruits to low temperature was related to antioxidative action of GA but the effect was dose-dependent with the peak after the treatment at 10 $g\ L^{-1}$. From the studies on other plants species, it is also known that antioxidative effect of exogenous GA is visible when the chemical is applied at concentration of 5 $g\ L^{-1}$ or higher (Addai et al. 2013; Ali et al. 2021) that is, at least 500 times more than used in our research. These results together with the outcomes of DPPH assay obtained in present study allow us to conclude that GA present in Gm at 10 $mg\ L^{-1}$ does not exert its stimulatory effect on the process by direct antioxidant action.

Numerous studies on human and animals revealed that GA can modulate the activity of cellular antioxidant system (e.g., Ahmed et al. 2016; Ayaz et al. 2017; Kadam et al. 2017) and this seems to be also reflected by our results. The total activity of POX determined at the 3rd and 7th day of anther culture was significantly higher on Gm than Cm. In addition, in the explants maintained for 7 days on Gm we identified higher number of active POX isoforms than in the anthers from Cm. POX are developmentally regulated enzymes which are involved in several physiological processes including cell wall formation and remodelling (Francoz et al. 2015). This cell wall-related activity of POX was suggested to be directly associated with different developmental events, such like meristemoid and meristem formation, callus growth or leaf and shoot development

(Kay and Basile 1987; Sharma et al. 2020). In case of androgenesis, Uvačkova et al. (2012) reported in maize a coincidence between intensification of microspore divisions and increased POXs activities during early stages of regeneration. Our previous studies revealed that MCSs resulting from sporophytic divisions of barley microspores occurred on Gm between 5 and 10 days of culture with the frequencies several-fold higher on Gm than Cm (Makowska et al. 2017). According to densitometric analysis performed here, the number of active POX isoforms as well as total activity of this enzyme gradually increased during culture on Gm, suggesting possible involvement of POX in efficient MCSs production on this medium.

It is also worthy to note, that the increase in total activity of POX on Gm paralleled the decline in endogenous concentration of H_2O_2 in the anthers, indicating involvement of this enzyme in regulation of H_2O_2 content as well as the progression of androgenesis. Indeed, in opposite to POX, the activity of another H_2O_2-consuming enzyme i.e., CAT did not differ between the anthers maintained on Gm and Cm. In the literature, there is no data on the influence of GA on CAT activity in plant cells, however, the studies performed on animal and human cells point to its strong stimulation by supplementation with GA (Mahmoud et al. 2011; Gado et al. 2013; Ahmed et al. 2016). CAT is one of the major enzymatic H_2O_2 scavenger and its role during morphogenesis in vitro was ascribed to the removing of H_2O_2 that is produced in excess under stressful culture conditions (Libik et al. 2005; Konieczny et al. 2014). The barley anthers freshly isolated from spikes and those after incubation in the pre-treatment solution displayed the highest CAT activity among material studied, which may indicate the possible protective role of this enzyme against stress associated with explant preparation and harmful pre-culture. However, when the anthers were plated onto culture media, the activity of CAT decreased as soon as after 3 days on Gm and Cm reaching about half of the level determined for those freshly isolated from spikes and those after stress pre-treatment. These observations are consistent with the results of several studies in which a sharp decrease in CAT activity was found to precede the expression of totipotency in various in vitro cultured plant species. (e.g., Cui et al. 1999; Gupta and Datta 2003/2004; Konieczny et al. 2008). In the highly responsive anthers from Gm, the decline in CAT activity paralleled an increase in POX activity and concomitant decrease in endogenous H_2O_2 concentration. In contrast, the anthers maintained on Cm retained relatively high level of endogenous H_2O_2 and displayed extremely low activity of POX throughout the culture. Thus, the anthers from Cm and Gm differ in the strategy against H_2O_2 overproduction, in that the POX activity in GA-exposed explants seems to substitute low activity of CAT as H_2O_2 scavenger which, in turn, is not observed in Cm-cultured material. The physiological meaning of the shift from CAT to POX-antioxidant activity in the anthers cultured on Gm remains unclear, however, the utilization of H_2O_2 for the POX-mediated cell wall formation and reconstruction during production and growth of MCSs cannot be ruled out.

In total protein extract isolated from the anthers we identified the activity of Cu/ZnSOD (two isoforms) and MnSOD (one isoform), whilst the activity of FeSOD was not detected. FeSOD is localized in chloroplasts (for review, see Alscher et al. 2002), so the lack of activity of this enzyme in the material studied can be accounted by the specificity of anthers which consists mostly of non-photosynthetic tissue and/or by culture conditions, i.e. the maintenance of the explants in continuous darkness.

Among SOD isoforms, the activity of MnSOD and Cu/ZnSOD-2 was enhanced in the presence of GA as revealed by densitometric analysis of isoenzymes pattern. SODs are engaged in the conversion of superoxide radical to molecular oxygen and hydrogen peroxide, which in turn is neutralized by POX. The coordinated action of SOD and POX were reported in maize to be crucial in regulation of the level of hydrogen peroxide involved in signaling during androgenic switch in anthers (Uvačkova et al. 2012). SOD activity was also correlated to androgenic responsivity in *Triticale* (Żur et al. 2009). MnSOD and Cu/ZnSOD have different cellular localization, in that MnSOD is mainly localized to mitochondrial and peroxisomes whilst Cu/ZnSOD is located in cytosol and extracellular matrix. The enhanced activity of these isoforms was found to accompany the intensification of catabolic processes like glycolysis, tricarboxylic acid cycle (TCA) and related to them the electron transfer/oxidative phosphorylation (for review see: Alscher et al. 2002). Previously we found that microspores maintained in the presence of GA started to produce MCSs after 3 days of culture, that is about 3–5 days earlier than those grown on a control medium (Makowska et al. 2017). MCSs formation, characterized by high cell division rate and fast growth, requires high amounts of energy. Thus, an increased supply of metabolites via glycolysis for the TCA and subsequent energy supply via the mitochondrial respiratory chains seems obvious. Interestingly, throughout the culture, the anthers maintained on Gm displayed several fold higher activities of mitochondrial enzymes, that is FUM and COX, than those cultured on Cm, which suggests intense catabolic activity of these explants. Thus, it cannot be ruled out that the high activity of the MnSOD and also Cu/ZnSOD-2 already at the 3 day of culture on GA medium is a response of explants to a higher energy demand and—which is directly related to it—an increase in the rate of catabolic processes in heterotrophic callus.

One of the questions arising after these experiments is whether GA-related changes in the activity of enzymatic antioxidants and H_2O_2 content are the reasons or the results

of androgenic induction. Considering the possible nutritional function of GA in androgenic development (Letarte et al. 2006) it may not be ruled out that GA-associated increase in the activity of antioxidant enzymes and enhancement of androgenesis is a result of the higher metabolic rate of explants, which in turn could result from utilization of additional (aside those present in the medium), GA-derived nutrients. However, another scenario, in which GA-associated efficient antioxidative protection was a prerequisite for the high metabolic rate needed for efficient androgenesis is also plausible. In the light of the experiments presented here, and those performed previously (Makowska et al. 2017), the role of GA in androgenesis seems to be complex and involves at least two different mechanisms: the first related to its indirect effect on antioxidative system of cultured cells and the second related to the presence of specific AGPs.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-023-02451-4.

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Author contributions RK and JZ designed the experiment. AK, KM, SO, MLK, MS, IG and WB performed the experiments and the data analysis. RK and AK prepared the manuscript. All authors approved the final version of the manuscript.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interests.

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