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Activities of antioxidant systems during germination of *Chenopodium rubrum* seeds

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Abstract

The activities of superoxide-dismutase (SOD), catalase (CAT) and peroxidase (POD), and concentrations of glutathione and ascorbate have been studied during the first stages of germination in *Chenopodium rubrum* L. seeds. The highest CAT and SOD activity was found prior to radicle protrusion, while POD activity was maximal at the time of radicle protrusion and seedling development, new POD isozymes simultaneously appearing. The concentrations of total, reduced and oxidized glutathione showed similar changes during germination, the highest values being detected at the time of radicle protrusion. Ascorbic acid was present in the seeds in a detectable concentration only at the time preceding radicle protrusion, while its oxidized form dehydroascorbic acid was detected during the whole germination period studied. Gibberellic acid (GA_3, 160 μM) had no effect on germination percentage, but in presence of GA_3, SOD and CAT activity notably increased prior to radicle protrusion, and oxidized glutathione concentration decreased in further germination.

Additional key words: ascorbic acid, catalase, gibberellic acid, glutathione, peroxidase, reactive oxygen species, superoxide-dismutase.

Introduction

Seed germination is a complex process requiring a multidisciplinary approach in analysis (Bewley and Black 1982). The sequence of the metabolic pattern that occurs during germination involves the activation of specific enzymes at the appropriate times and regulation of their activity. The final result of this process is radicle protrusion and seedling formation (Roberts 1972, Riley 1987).

There is a growing interest in the functional role of reactive oxygen species (ROS) and corresponding scavenging enzymic and nonenzymic systems in seed germination. Appearance of ROS in the plant cells is generally linked with the free radical processes involved in the plant developmental processes, as well as its interaction with the environment (Elstner 1982, Hendry and Crawford 1994). Free radicals are continuously damaging a multitude of molecular species, being able even to destroy cell membranes and organelles. On the other hand, some of them, such as H_2O_2 and $O^{\cdot}$, are proposed to have a signalling role in the cell during stress action (Baker and Orlandi 1995, Lamb and Dixon 1997). Organic free radicals and ROS are alternately produced and removed. Antioxidant enzymes such as superoxide-dismutase, peroxidase and catalase are considered to be the main protective compounds engaged in the removal of free radicals and activated oxygen species (Van Loon 1986, Bowler *et al.* 1992, Khan and Panda 2002). Water-phase antioxidants glutathione and ascorbate have a general protective role against oxidative stress, by regulating redox equilibrium in the cell (Arrigoni 1994, May *et al.* 1998). It is suggested that ascorbic acid/dehydroascorbic acid ratio could block or delay cell division (de Pinto *et al.* 1999).

Different explanations of the effect and role of ROS

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Abbreviations: AA - ascorbic acid; CAT - catalase; DHA - dehydroascorbic acid; DTNB - 5, 5'-dithio-bis(2-nitrobenzoic acid); EDTA - ethylenediaminetetraacetic acid; GA_3 - gibberellic acid; GSH - reduced glutathione; GSSG - oxidized glutathione; POD - peroxidase; SOD - superoxide-dismutase

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in the seed germination have been considered. ROS production in seeds has mostly been considered as an event causing oxidative stress with negative consequences (Hendry 1993, Gidrol *et al.* 1994). However, some authors have shown that the production of ROS during seed germination may be a beneficial biological reaction, linked with high germination capacity, seedling development and protective function against parasitic organisms during germination (Schopfer *et al.* 2001).

Gibberellins, usually GA_3, GA_4 and GA_7, are successful in breaking dormancy in numerous species and also in accelerating germination of non-dormant seeds (Bewley and Black 1982, Riley 1987), while in some species are ineffective (Moore *et al.* 1994, Boscagli and Sette 2001). The data about effect of GA_3 on ROS metabolism are not abundant. Schopfer *et al.* (2001) have shown that, in case of radish germination, inhibition of ROS production caused by far-red light and abscisic acid is reversed by GA_3. Bethke *et al.* (2000) reported that GA_3-treated barley aleurone protoplasts are less able to metabolize ROS. Jones (1986) found that GA_3 stimulates production of peroxidase in barley aleurone.

Chenopodium rubrum L., qualitative short-day plant, is a suitable model for stydying photoperiodic and hormonal requirements for growth and flowering (Ullmann *et al.* 1980, Živanović *et al.* 1995).

In this work we studied the change in the concentration of antioxidant compounds: glutathione, ascorbate, and activities of antioxidative enzymes: peroxidase (POD), catalase (CAT) and superoxide-dismutase (SOD), during *C. rubrum* seed germination, and the effect of GA_3 on these parameters. Our aim was: to follow the expression of particular parts of anti-oxidative systems in the seeds during the early stages of germination, to see whether GA_3 has any effect on these processes, to find out if there is a time correlation between concentration of antioxidative compounds and activities of antioxidative enzymes with particular phases of germination.

Materials and methods

Plants and cultivation: Three-year-old seeds of *Chenopodium rubrum* L., ecotype 184 were used. Plants for seed propagation were grown under natural daylight and photoperiod, from April to August, in pots filled with 1:2 sand and peat. Temperature was 30 ± 5 °C. The seeds were stored at room temperature until used.

These seeds were sown in 8.5 cm diameter Petri dishes containing 5 cm^3 distilled water or GA_3 (160 μM) solution and germinated under 24 h dark at 32 °C, 24 h dark at 10 °C, and 48 h light at 32 °C (irradiance of 70 μmol m^{-2} s^{-1}). Germination percentage was determined, in batches of 100 seeds per sample, using protrusion of the radicle by more than 2 mm as a criterion (Schopfer and Plachy 1984).

Extraction of seeds: Whole seeds/seedlings (obtained from 0.05 g of dry seeds per sample) were powdered in liquid nitrogen. Frozen seed powder was added to 2 cm^3 extraction buffer containing 0.25 M sucrose, 0.05 M Tris and 1 mM EDTA, pH 7.4. The mixture centrifuged for 15 min at 900 *g*. The supernatant was used for POD, CAT, SOD activity and glutathione measurements.

Enzyme assays: SOD activity was determined spectrophotometrically measuring the percent of the SOD-induced inhibition of adrenalin autooxidation at the alkaline pH (Misra and Fridowich 1972), in a total volume of 3 cm^3. Adrenalin stock solution was freshly prepared before the measurement and contained 0.3 mM adrenalin in 0.1 M HCl. The assay mixture contained 0.05 M sodium carbonate buffer pH 10.2, 0.1 mM EDTA, 0.1 cm^3 adrenalin stock solution and variable amounts of enzyme preparations. Adrenalin autooxidation was monitored using *Shimadzu UV-160* (Kyoto, Japan) spectrophotometer at 480 nm, during 10 min at 26 - 30 °C.

POD activity was determined spectrophotometrically with guaiacol as the substrate in a total volume of 3 cm^3. The assay mixture contained 50 mM acetate buffer pH 5.5, 92 mM guaiacol, 18 mM H_2O_2 and variable amounts of the enzyme preparations. The turnover of guaiacol was monitored at 470 nm. Reaction rate was calculated from the coefficient of absorbance for guaiacol of 25.5 $mmol^{-1}$ cm^{-1}.

CAT activity was determined using a Clark-type polarographic electrode at 28 °C. Seed extract (0.02 cm^3) was added to 0.96 cm^3 0.1 M Tris pH 7.6 and oxygen release was initiated by 0.02 cm^3 0.1 M H_2O_2. The rate of O_2 release was calculated and expressed as the amount of O_2 released per min and mg of protein.

Protein concentration of the seed extracts was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Glutathione detemination: Total glutathione was determined by enzymatic recycling assay as described by Griffith (1980). Seed extract was mixed with double volume of 5 % 5-sulphosalicylic acid and centrifuged for 10 min at 2 500 *g*. Three working solutions were made up in stock buffer (125 mM potassium phosphate, 6.3 mM EDTA, pH 7.5): (I) 0.3 mM NADPH, (II) 6 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and (III) 5 units of glutathione reductase per cm^3. For total glutathione assay, 0.7 cm^3 of solution I, 0.1 cm^3 of solution II and 0.2 cm^3 of the extract were mixed.

Reaction was started with 0.01 cm^3 of solution III and followed at 30 °C by measuring absorbance change at 412 nm during 2 min. Oxidized glutathione (GSSG) was determined by derivatization of thiol group in reduced glutathione (GSH) with 2-vinylpyridine in slightly acidic solution. GSH was determined by the difference of the two values.

Ascorbate (AA) and dehydroascorbate (DHA) extraction and assay: Whole seeds/seedlings (obtained from 0.05 g of dry seeds per sample) were frozen in liquid nitrogen, ground to a powder, and extracted in 1 cm^3 5 % perchloric acid. The homogenate was centrifuged for 10 min at 12 000 *g*, and the supernatant was neutralized with 5 M potassium carbonate to pH 5.6. The neutralized supernatant was centrifuged for 2 min at 12 000 *g* and ascorbate was estimated immediately.

AA was measured in the extract by the decrease in absorbance at 265 nm (Hewitt and Dickes 1961) upon the addition of ascorbate oxidase (1 U) from *Cucurbita* (*Sigma*, St. Louis, USA) to an assay mixture containing 0.1 M potassium phosphate buffer (pH 5.6) and extract in a final volume of 3 cm^3. DHA was measured as AA after reduction using dithiothreitol (DTT). The reaction started by the addition 0.02 cm^3 of 1 mM DTT to the assay mixture containing 0.15 M potassium phosphate buffer pH 7.6 and extract in a final volume of 3 cm^3. AA and DHA concentration was calculated using the coefficient of absorbance for ascorbate at 262 nm (14.3 $mmol^{-1}$ cm^{-1}).

Isoelectric focusing of soluble POD: Soluble POD isozymes were separated in a pH gradient from 3 to 9 (using 3 % ampholite solution) on a 7.5 % polyacrylamide gel. The isozymes were stained on gel with 0.03 mM 4-chloro-1-naphtol and 5 mM H_2O_2 in Na-acetate buffer pH 5.5 for 10 min at 25 °C. Total proteins were detected on the gel by staining with Coomassie Brilliant Blue R250.

Statistical analysis of data: Each experimental variant and control was represented by four replicates. Statistical analysis of the data was performed using the Mann-Whitney U ranking test, at the 0.05 level of significance. Student *t* - test was applied for the statistical analysis of germination percentage.

Results and discussion

Seed germination: Radicle protrusion occurred on the third day. In subsequent days, germination percentage increased significantly. The presence of GA_3 (160 μM) inhibited seed germination, but not significantly (Fig. 1). In some other seed species (*Satureja montana*, *Pottosporum eugenioides*, *P. tenuifolium*, *P. crassifolium*) GA_3 (1.6 μM - 1 mM) treatment was also ineffective in germination (Moore *et al.* 1994, Boscagli and Sette 2001).

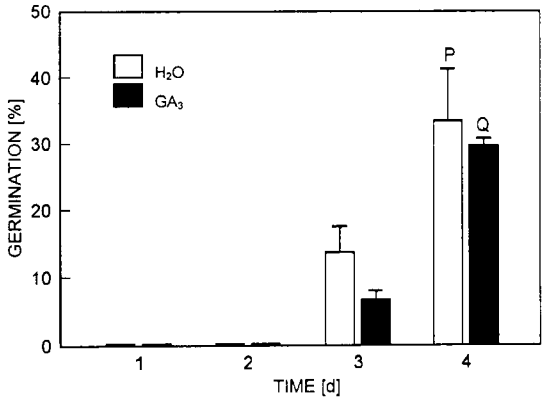

Fig. 1. Time course of *C. rubrum* germination on water (H_2O) or GA_3 solution (160 μM); P,Q - statistically significant differences ($P < 0.05$) from the third day.

Activity of CAT, SOD and POD during germination: Protein content, as well as CAT, SOD and POD activity, varied in a similar way in untreated and GA_3 treated seeds (Figs. 2, 3). Protein concentration increased in untreated seeds during germination, since proteins are both released from protein storage or synthesized *de novo* after imbibition, as building and regulatory material in the emerging seedling (Roberts 1972). In GA_3 treated seeds protein concentration decreased significantly on the 2nd day, while on the 3rd and 4th day was significantly higher in comparison with the 1st day (Fig. 2). The isoelectrophoregram of the total proteins also shows an increase in both acidic and basic protein content during germination (Fig. 4 a), this change being more pronounced in the case of acidic ones. The bands with pI around 4 and 8.8 are present during the whole germination period. On the 4th day of germination the number of protein bands is significantly higher in comparison with the previous days, in both untreated and GA_3 treated seeds. The new bands appear between pI 4.6 and 5.1.

In untreated seeds there was no significant variation in CAT activity during germination. SOD activity decreased during germination, on the 4th day being significantly lower in comparison with the first day. In GA_3 treated seeds, there was a significant increase in both CAT and SOD activity on the 2nd day of germination, and a decrease in SOD activity on the 4th day (Fig. 3*A,B*) comparing to the first day. Comparing GA_3 treated and untreated seeds, significant increase in both CAT and SOD activity in treated seeds was detected on the 2nd day.

POD activity was not detectable spectrophotometrically during the first two days in both untreated and

GA_3 treated seeds (Fig. 3). On the 4th day there was a significant increase in POD activity in comparison with the 3rd day, in both untreated and treated seeds (Fig. 3*C*). There was no statistically significant difference in POD activity between untreated and GA_3 treated seeds. On the zymogram of the soluble PODs, both anionic and cationic isozymes were detected. In the group of the anionic PODs, the isoenzyme with pI value around 4 is present during the whole germination period, while two isozymes with pI values around 4.6 appear on the 4th day. The cationic isoenzyme with pI value around 8.5 is constantly present after imbibition, and an isoenzyme with pI value around 8.8 appears on the 4th day (Fig. 4*B*). There was no difference between POD zymograms of GA_3 treated and untreated seeds.

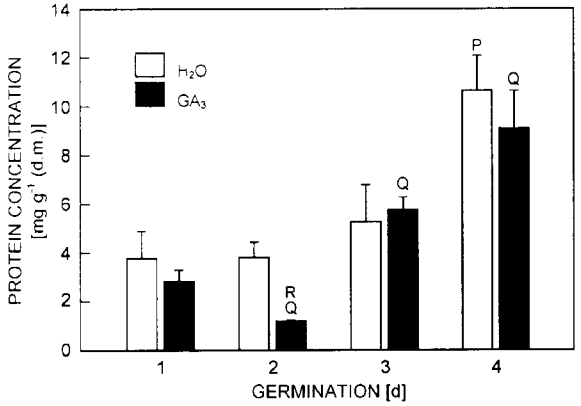

Fig. 2. Protein concentration during *C. rubrum* germination on water (H_2O) or GA_3 solution (160 μM); P,Q - statistically significant differences ($P < 0.05$) from the first day, R - statistically significant difference ($P < 0.05$) between treated and untreated seeds.

The obtained results show that CAT and SOD activity peaks preceded POD maximal activity. CAT and SOD showed the highest activity at the time preceding radicle protrusion (Figs 1, 3), while the significant expression of POD occurred after this term. Gidrol *et al.* (1994) observed a SOD activity peak during early imbibition of soybean seeds, followed by a CAT and POD activity peak. Though GA_3 was ineffective in seed germination (Fig. 1), it exerted an influence on particular antioxidative parameters in certain germination stages. Significant increase in CAT and SOD activity (Fig. 3*A*,*B*) and corresponding decrease in protein content (Fig. 2) in GA_3 treated seeds in comparison with untreated ones, on the second day of germination, may show that this hormone interacts with the metabolism of H_2O_2 and $O_2^{\cdot -}$ at the time preceding radicle protrusion.

POD showed a different kinetics of expression in germination, in comparison with CAT and SOD (Fig. 3). Though POD activity was not detectable spectrophotometrically during the first two days, one acidic and one basic POD isozyme could be detected on the zymogram during the whole germination period (Fig. 4*B*), due to the higher sensitivity of this method comparing to the spectrophotometric assay. Significant increase in POD activity on the 4th day of germination (Fig. 3*C*) corresponded to the expression of the new isozymes on the zymogram (Fig. 4*B*). Morohashi (2002) reported that in tomato seeds POD activity developed prior to radicle protrusion and increased notably afterwards. Mitchell and Barrett (2000) found three types of seed and seedling PODs when analyzed on zymograms, which were

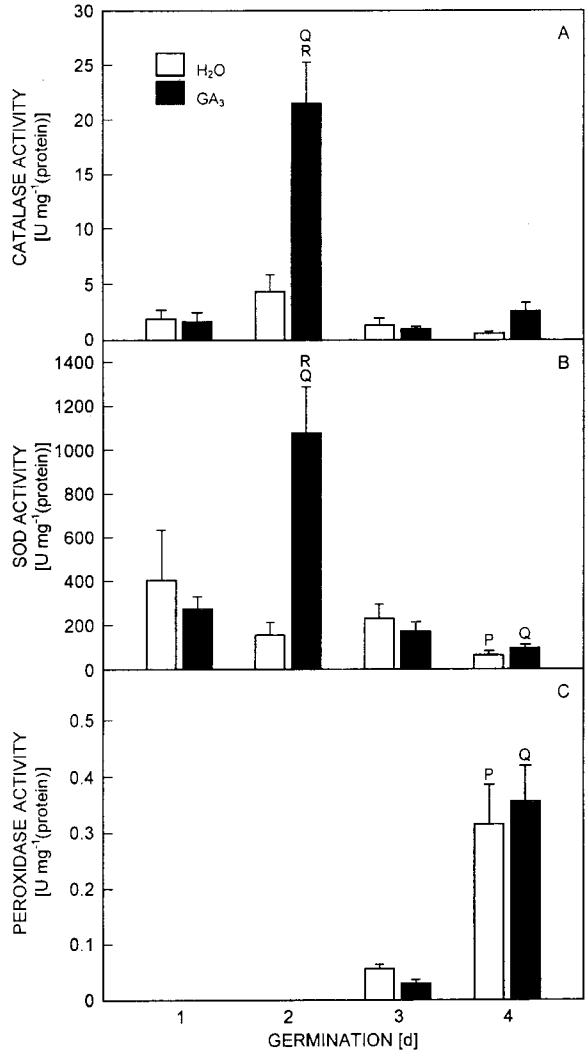

Fig. 3. Catalase (*A*), superoxide-dismutase (*B*) and peroxidase (*C*) activities during *C. rubrum* germination on water (H_2O) or GA_3 solution (160 μM); P,Q - statistically significant differences ($P < 0.05$) from the first day (in case of POD from the 3rd day), R - statistically significant difference ($P < 0.05$) between treated and untreated seeds.

expressed gradually during germination of *Viola carnuta* seeds. Our results show that POD isozymes develop gradually during germination of *C. rubrum* seeds, and the increase in POD activity after radicle protrusion is

connected with *de novo* production of the new isozymes. The fact that there was no difference in POD isoelectrophoretic pattern between untreated and treated seeds shows that GA_3 did not affect synthesis of the new isozymes. Our results are in agreement with the results of Schopfer *et al.* (2001), which have shown that during germination of radish seeds POD activity appears at the time of radicle emergence and is considerably increased in the period of the seedling development. They also found that 160 μM GA_3 shifts the period of maximal POD concentration on the time scale.

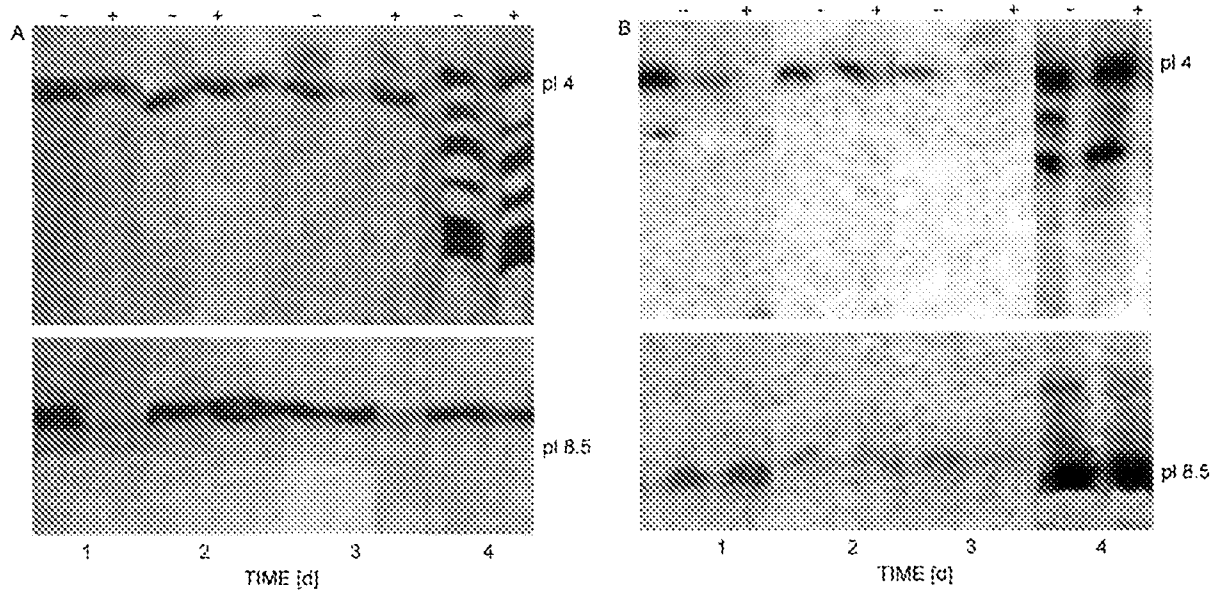

Fig. 4. Isoelectric focusing of proteins (*A*) and peroxidase isozymes (*B*) during *C. rubrum* germination. Each lane contains 0.02 cm^3 of the extract. The figure is composed from representative lanes of several gels; (-), (+) - untreated and GA_3 treated seeds, respectively.

Glutathione and ascorbate: In first two days of germination there was no significant variation in the concentrations of GSH, GSSG and total glutathione, both in untreated and GA_3 treated seeds. On the third day there was a notable increase in all three parameters, followed by a significant decrease on the 4th day (Fig. 5). On the 4th day GA_3 induced a statistically significant decrease in GSSG concentration in comparison with untreated seeds (Fig. 5*A*). Over 80 % of the glutathione pool was present in the reduced state. The obtained results show that the glutathione system in the germinating seeds followed a different developmental pattern (Fig. 5) in comparison with the activity of antioxidative enzymes (Fig. 3). The maximal content of total glutathione, as well as of its reduced and oxidized form, was attained on the third day after imbibition both in untreated and GA_3 treated seeds, corresponding to the radicle protrusion. Tommasi *et al.* (2001) have also observed an increase in glutathione during the first 24 h of germination of pine seeds, which was followed by its decrease. The GSSG/GSH ratio may be a sensitive indicator of oxidative stress (May *et al.* 1998). Higher concentration of GSH than GSSG in germinating *C. rubrum* seeds is an indicator of the high antioxidative capacity. According to the results, GA_3 did not show any significant effect on glutathione system in treated seeds in comparison with untreated ones. The significantly lower GSSG concentration in GA_3 treated seeds on the 4th day of germination (Fig. 5*A*) may be a consequence of the lower production of free radical species in the phase following radicle protrusion.

Ascorbate was detected only on the 2nd day of germination, prior to radicle protrusion, GA_3 inducing a decrease in the AA content (Fig. 6*A*). DHA was present throughout the observed germination period. Again, in the case of DHA there was also a reduction in concentration on the 2nd day of germination in the case of GA_3 treated seeds (Fig. 6*B*). Though, the observed difference on the 2nd day of germination in both the case of AA and DHA, was not statistically significant (Fig. 6). Other authors did not detect AA at the beginning of the imbibition either. However, in subsequent germination phases (Tommasi *et al.* 2001), or just before radicle protrusion (Pallanca and Smirnoff 1999), a rise in AA concentration was also observed.

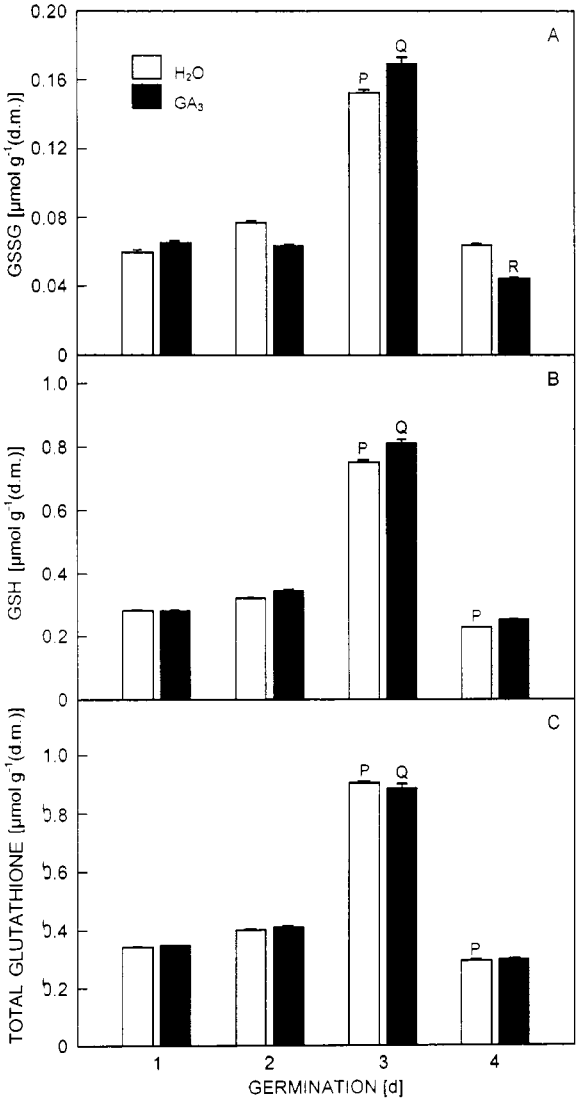

Fig. 5. Content of GSSG (*A*), GSH (*B*) and total glutathione (*C*) during *C. rubrum* germination on water (H_2O) or GA_3 solution (160 μM); P, Q - statistically significant differences ($P < 0.05$) from the first day, R - statistically significant difference ($P < 0.05$) between treated and untreated seeds.

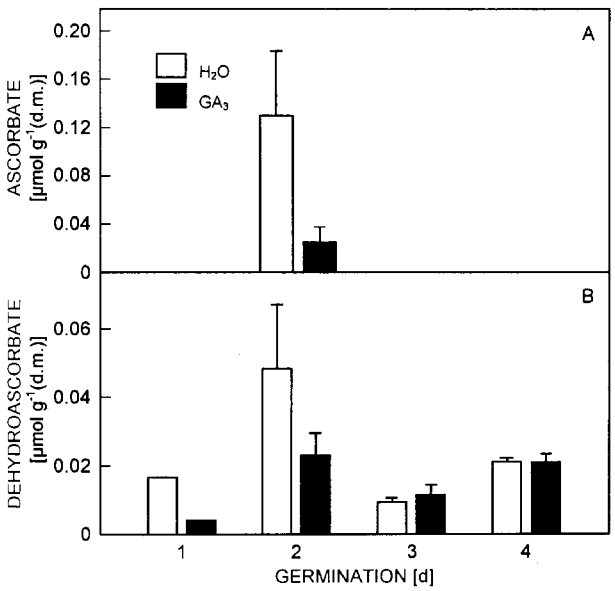

Fig. 6. Content of ascorbate (*A*) and dehydroascorbate (*B*) during *C. rubrum* germination on water (H_2O) or GA_3 solution (160 μM).

Our results indicate a sequential expression of the antioxidative system during *C. rubrum* germination. Further studies are necessary to determine the role of its particular parts. The effect of various GA_3 concentrations is also to be studied.

To conclude, our results demonstrate that GA_3 (160 μM) inhibited seed germination, but not significantly. GA_3 induced a rise in the activities of catalase and SOD on the 2nd day of germination prior to radicle protrusion, with a concomitant decrease in the concentration of AA and DHA. Glutathione and peroxidases showed an increase in the latter stages in both the treated and control plants, oxidized form of glutathione being significantly decreased on the 4th day of germination.

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