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BRIEF COMMUNICATION

# Changes in activities of antioxidant enzymes during *Chenopodium murale* seed germination

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## Abstract

The activities and isoenzyme pattern of catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) have been studied during germination of *Chenopodium murale* seeds. CAT and SOD activities were similar in dry seeds and during first 2 d of imbibition. CAT activity increased during radicle protrusion and early seedling development. The maximum SOD activity was found at final stages of germination and early seedling development. POD activity was not detected until the 6$^{th}$ day of germination, indicating POD involvement not until early seedling development. Gibberellic acid ($GA_3$, 160 μM) delayed and synchronized *C. murale* germination.

*Additional key words*: catalase, gibberelic acid, peroxidase, superoxide dismutase.

Seed germination starts with imbibition, and ends with radicle protrusion. It can be divided in three phases: imbibition (rapid initial water uptake – physical process characteristic also for dead seeds), the plato phase (small change of water content but high metabolic activity) and further water uptake coinciding with radicle protrusion and growth (Giba *et al*. 2004). Plato phase, as the most important in regulation of germination, involves the activation of specific enzymes at the appropriate time and regulation of their activity (Riley 1987). Seed germination is a complex process, associated with many metabolic, cellular and molecular events. It was supposed that accumulation of reactive oxygen species (ROS), during seed imbibition, leads to germination (Bailly 2004). $H_2O_2$ is produced at the early imbibition period in soybean seeds (Puntarulo *et al*. 1988). Accumulation of other ROS, such as hydroxyl and superoxide radicals (Schopher *et al*. 2001) also occurs during seed germination of radish. The production of ROS by germinating seeds has often been regarded as a cause of stress. Therefore, antioxidant enzymes have been considered to be of particular importance for the completion of germination. Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) are considered to be the main protective enzymes, being engaged in the removal of free radicals and activated oxygen species (Blokhina *et al*. 2003). These detoxifying enzymes also have important roles throughout plant ontogeny, from seed germination (Bailly 2004) to growth and development (Procházková and Wilhelmová 2004, Synková *et al*. 2006). In germinating sunflower seeds, CAT activity increased prior to radicle protrusion, the latter being concomitant with the elimination of $H_2O_2$ (Bailly *et al*. 2000). Stimulation of the activity of SOD during these processes has also been reported (Puntarulo *et al*. 1991).

Gibberellins play an important role in stimulation of seed germination (Bewley 1997). They are efficient in breaking seed dormancy, by their ability to overcome the requirements for environmental factors, and in accelerating germination in non-dormant seeds (Bewley and Black 1982). In some species, they are ineffective (Moore *et al*. 1994, Boscagli and Sette 2001). In *Chenopodium rubrum* $GA_3$ (160 μM) slightly inhibited germination (Dučić *et al*. 2003/4) and in *Coffea arabica* $GA_{4-7}$ (1 - 1000 μM) inhibited germination and caused cell death in the embryo (Da Silva *et al*. 2005).

*Chenopodium murale* L. is an herbaceous non-rosette annual. Ecotype 197, a facultative long-day plant (Cumming 1967) is a suitable model plant for studying

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*Abbreviations*: CAT - catalase, EDTA - ethylenediaminetetraacetic acid, $GA_3$ - gibberellic acid, POD - peroxidase, ROS - reactive oxygen species, SOD - superoxide dismutase, TEMED - N,N,N',N'-tetramethylethylenediamine.

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photoperiodic and hormonal requirements for growth and flowering (Pavlová *et al*. 1989, Mitrović *et al*. 2000). The use of long-day species of genus *Chenopodium* of a similar type of morphogenesis, as short-day ones, may be important for comparative studies (Pavlová *et al*. 1989). Our results indicated a sequential expression of anti-oxidative enzymes during *C. rubrum* seed germination (Dučić *et al*. 2003/4).

In this work, we studied the activities and isoenzyme pattern of antioxidative enzymes CAT, SOD and POD during *C. murale* seed germination. Our aim was to follow the expression of particular parts of antioxidative systems during germination, to study the effect of $GA_3$ on these processes and to find the correlation between activities of these antioxidative enzymes with particular phases of *C. murale* germination.

Plants for seed propagation were grown in the greenhouse of Institute for biological research Siniša Stanković, in Belgrade (44° 49′ N, 20° 29′ E) under natural photoperiod and temperature, from April to August, in 1:2 sand and peat. Three-year-old seeds of *Chenopodium murale* L. ecotype 197 were used. For each experimental point, 4 replicates of 50 seeds (0.0305 ± 0.0002 g) were sown in Petri dishes containing 5 $cm^3$ distilled water or $GA_3$ (160 μM) solution. Germination was attained by temperature and dark/light cycles (48 h light at 30 °C, followed by 24 h darkness at 5 °C, 24 h light at 30 °C, 24 h darkness at 5 °C and 24 h light at 30 °C; irradiance of 70 μmol $m^{-2}$ $s^{-1}$). At the end of each cycle, germination was scored, and seed/seedlings were frozen in liquid nitrogen until the extraction. As a criterion of germination, radicle protrusions by more than 2 mm were used (Schopfer and Plachy 1984).

Dry seeds, 2.5 h imbibed seeds and 48 - 144 h imbibed in distilled water or $GA_3$ seeds/seedlings, were used in the experiments. For each experimental point, four replicates of 50 seeds/seedlings were powdered in liquid nitrogen. Extraction buffer contained 0.25 M sucrose, 0.05 M Tris pH 7.4 and 1 mM EDTA. Frozen seeds/seedlings powder was added to the extraction buffer in the 1:30 ratio. The mixtures were centrifuged 10 min at 10 000 *g*, and obtained supernatants were used for determination of CAT, SOD and POD activity and protein concentration.

CAT, SOD and POD activities were determined spectrophotometrically using a *Shimadzu UV2501 PC* (Tokyo, Japan) spectrophotometer. SOD activity was recorded at 550 nm using xantine/xantine oxidase system as described by McCord and Fridovich (1968). One unit of SOD activity was defined as the amount of enzyme that causes 50 % inhibition of cytochrome *c* reduction. CAT activity was measured by recording decrease in absorbance of $H_2O_2$ at 240 nm, by the method of Bergmeyer (1983). POD activity was monitored at 470 nm using guaiacol as the substrate according to the modified method of Chance and Maehly (1956). The assay mixture contained 50 mM sodium acetate buffer (pH 5.5), 92 mM guaiacol, 18 mM $H_2O_2$ and variable amounts of enzyme at 25 °C. Reaction rate was calculated from the coefficient of absorbance for tetraguaiacol of 25.5 $mM^{-1}$ $cm^{-1}$. One unit of CAT and POD activity was defined as the amount of enzyme that converts 1 μmol of substrate to product in 1 min. Protein concentration of the extracts was deter-mined by the method of Bradford (1976) with bovine serum albumin as the standard.

Native polyacrylamide gel electrophoresis of CAT was carried out under nondenaturing conditions in gels containing 8 % polyacrylamide. A constant current of 25 mA per gel was applied for 2 h. Electrophoresis buffer and gels were prepared as described by Laemli (1970) except for SDS being excluded. Equal volumes of all samples were loaded on the gels. CAT was visualized on gel by the method of Woodbury *et al.* (1971).

Non-equilibrated isoelectric focusing was performed horizontally in the *LKB 2117 Multiphor II* (*Pharmacia Biotech*, Uppsala, Sweden) system, using 0.5 mm thick polyacrylamide gels (5 % T, 3 % C), containing 4 % 3.5 - 10.0 ampholites. Gels were run for 2 h at 5 °C with the constant power of 0.25 W $cm^{-1}$ width of gel, with limiting voltage of 2000 V. SOD isoenzymes were detected on the gels by the method of Beauchamp and Fridovich (1971). In order to distinguish between Mn, Cu/Zn and Fe-SOD isoforms, activity staining was performed in the gels previously incubated for 40 min in 100 mM sodium phosphate buffer pH 7.8 containing 5 mM KCN or 5 mM $H_2O_2$. POD was stained on gel after the method of Lagrimini and Rothstein (1987).

Statistical analysis of data was performed using *ANOVA* test, at the 0.05 level of significance.

*C. murale* radicle protrusion occurred as early as 2nd day of imbibition (Table 1), both in distilled water (11 %) and in $GA_3$ (160 μM) solution (1 %). $GA_3$ inhibited germination from 1st to 5th day of imbibition, while on the 6th day of imbibition, germination in distilled water and $GA_3$ solution was about the same (96 and 92 %, respectively). We showed previously that $GA_3$ (160 μM) inhibited *Chenopodium rubrum* germination during first 4 d of imbibition (Dučić *et al.* 2003/4). When imbibed in distilled water, most of (about 70 %) *C. murale* seeds germinated from 3rd to 4th day of imbibition (16 - 85.5 %). $GA_3$ (160 μM) delayed germination and the most of $GA_3$ treated seeds (40 %) geminated from 5th to 6th day (50.5 - 90.5 %). $GA_3$ synchronized *C. murale* germination, the most of the seedlings being at the same phase of development on the 6th day of imbibition (about 1 cm long root and hypocotyl, with seed coat still over cotyledons). Also, $GA_3$ lowered the difference between the rates of germination of two *C. murale* seed populations (Table 1).

CAT activity was on the same level in dry seeds and during first 48 h of imbibition in untreated and $GA_3$ treated seeds (Table 1). Significant increase in CAT activity of untreated seeds/seedlings was shown from the 3rd day of imbibiton, which coincides to intensive radicle protrusion (16 - 85.5 % of germination), reaching

maximum on the 6th day (Table 1). In $GA_3$ treated seeds, CAT activity was significantly lower compared to untreated seeds from 3rd to 6th day of imbibition. Catalase activity in $GA_3$ treated seeds remained at the same level during first 120 h of imbibition as in the dry seeds. Significant increase in CAT activity was observed on the 6th day of imbibition, which also corresponds to the significant increase in germination of $GA_3$ treated seeds (50.5 - 90.5 %). CAT activity of $GA_3$ treated seeds/ seedlings on the 6th day of germination reached the same level as untreated seeds on the 4th day, confirming the connection between the raise in CAT activity and *C. murale* radicle protrusion. We showed (Dučić *et al.* 2003/4) that CAT activity in untreated *C. rubrum* seeds did not change significantly during early stages of germination, while a peak in CAT activity of $GA_3$ treated seeds preceded radicle protrusion. CAT role was shown during sunflower seed desiccation by preventing dehydration-related oxidative damage (Bailly *et al.* 2004) and in detoxification of $H_2O_2$ generated by reserve lipid mobilization during germination (Bewley and Black 1994).

We obtained two close bands of CAT activity on native polyacrilamyde gel in all samples. The new band (Fig. 1*A*) appeared in extracts of untreated seedlings on the 6th day of germination, indicating that the new isoform is connected with early phases of *C. murale* seedling development (on the 6th day of germination, most of untreated *C. murale* seedlings are with fully developed cotyledons). These results are in accordance

Table 1. Germination [%], catalase, superoxide-dismutase and peroxidase activities [U $g^{-1}$ (d.m.)] and protein content [mg $g^{-1}$ (d.m.)] during *Chenopodium murale* seed germination (0 to 144 h) on distilled water or $GA_3$ (160 μM) solution. Means ± SE of four replicates of 50 seeds each.

| Parameter | Treatment | 0 h | 2.5 h | 48 h | 72 h | 96 h | 120 h | 144 h |
|---|---|---|---|---|---|---|---|---|
| Germination | $H_2O$ | - | 0 | 11.00± 1.3 | 16.00± 0.8 | 85.50± 2.8 | 85.00± 3.8 | 96.00± 0.8 |
| | $GA_3$ | - | 0 | 1.00± 0.6 | 1.50± 1.0 | 36.50± 4.0 | 50.50± 1.8 | 92.00± 2.7 |
| CAT | dry seeds | 1184.86±89.01 | - | - | - | - | - | - |
| | $H_2O$ | - | 1279.61±10.91 | 1324.20±70.38 | 1702.57±75.53 | 3147.32±171.5 | 3760.39±204.2 | 5137.02±154.3 |
| | $GA_3$ | - | 0 | 1412.75±187.9 | 1462.91±68.08 | 1802.27±92.09 | 1857.39±140.8 | 2835.41±293.3 |
| SOD | dry seeds | 321.66±19.28 | - | - | - | - | - | - |
| | $H_2O$ | - | 342.66± 5.14 | 310.52±26.90 | 426.66±20.74 | 601.97±14.87 | 665.29±45.36 | 494.97±31.25 |
| | $GA_3$ | - | 0 | 348.34±36.15 | 397.97±31.51 | 436.70±32.72 | 441.92±20.63 | 679.93±94.95 |
| POD | dry seeds | 0 | - | - | - | - | - | - |
| | $H_2O$ | - | 0 | 0 | 0 | 0 | 0 | 28.48± 2.54 |
| | $GA_3$ | - | 0 | 0 | 0 | 0 | 0 | 0 |
| Protein | dry seeds | 18.92± 1.10 | - | - | - | - | - | - |
| | $H_2O$ | - | 18.88± 0.60 | 20.88± 1.11 | 23.61± 0.51 | 32.75± 0.65 | 33.97± 0.54 | 36.79± 0.60 |
| | $GA_3$ | - | 0 | 22.35± 0.79 | 20.27± 0.75 | 28.77± 0.33 | 28.18± 0.46 | 33.67± 0.64 |

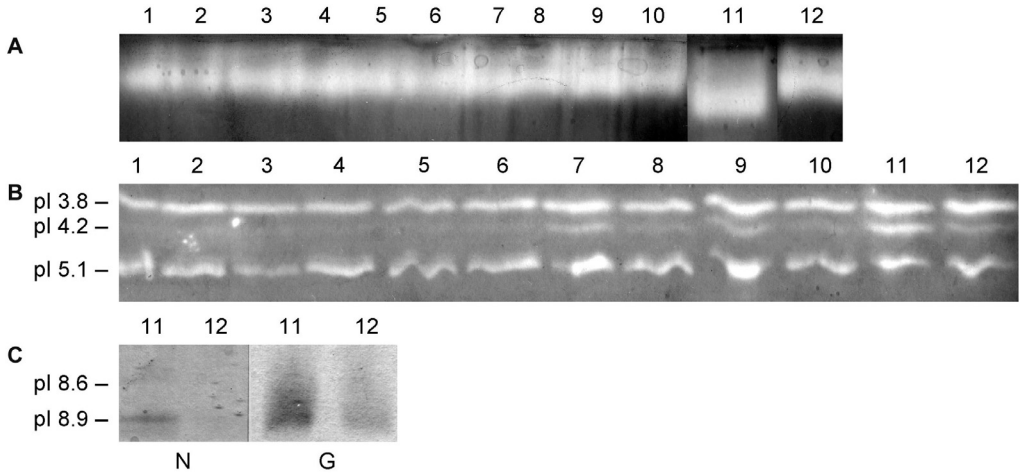


Fig. 1. Isoenzyme pattern of catalase after native electrophoresis (*A*), and SOD (*B*) and POD (*C*) after isoelectrofocusing, during germination of *C. murale* seeds, on $H_2O$ or $GA_3$ (160 μM). *Lane 1:* 0 h, dry seeds; *lane 2*: 2.5 h, $H_2O$; *lane 3*: 48 h, $H_2O$; *lane 4*: 48 h, $GA_3$; *lane 5*: 72 h, $H_2O$; *lane 6*: 72 h, $GA_3$; *lane 7*: 96 h, $H_2O$; *lane 8*: 96 h, $GA_3$; *lane 9*: 120 h, $H_2O$; *lane 10*: 120 h, $GA_3$; *lane 11*: 144 h, $H_2O$; *lane 12*: 144 h, $GA_3$. G - guaiacol, N - 4-chloro-1-naphtol.

with spectrophotometrically obtained data (Table 1). Niknam *et al*. (2006) observed one band of CAT activity in seedlings of *Trigonella* sp.

Similarly to CAT activity, SOD activity did not change during first 2 d of imbibition in untreated, and during 5 d in $GA_3$ treated seeds (Table 1). In untreated seeds SOD activity increased significantly from 3rd day, reaching maximum on the 5th day, corresponding to radicle protrusion in about 70 % of germinated *C. murale* seeds on the 4th day (16 - 85.5 %). SOD activity decreased significantly on the 6th day of germination (fully developed cotyledons). In $GA_3$ treated seeds (Table 1), maximum in SOD activity was detected on the 6th day of germination, which also corresponds to radicle protrusion in about 40 % of $GA_3$ treated *C. murale* seeds (50.5 - 90.5 %). Maximum in SOD activity in $GA_3$ treated seeds/seedlings (6th day) was delayed compared to SOD activity in untreated seeds/seedlings, similarly to their delay in germination (Table 1).

Three SOD isoforms were observed in all samples, with pI values 3.8, 4.2, 5.1 (Fig. 1*B*). The band with pI value around 4.2 was more intensive after 3rd day of imbibition, both in untreated and $GA_3$ treated seeds. Based on the specific inhibition of SOD activity by KCN and $H_2O_2$, we found that *C. murale* seeds/seedlings contain only Mn-SOD form.

In contrast to CAT and SOD activities, POD activity was not detectable spectrophotometrically in untreated seedlings until 6th day of germination, which is related to the early seedling development. No POD activity was detected spectrophotometrically in $GA_3$ treated seeds/ seedlings extracts (Table 1). In tomato (Morohashi 2002), radish (Schopfer *et al*. 2001) and *C. rubrum* (Dučić *et al*. 2003/4) seeds, POD activity appeared prior to or simultaneously with radicle protrusion, and increased during seedling development.

On the zymogram of PODs obtained by incubation in both guaiacol and 4-chloro-1-napthol, two basic isoenzymes with pI values 8.6 and 8.9 were detected. These bands were visible only on the 6th day, in both untreated and $GA_3$ treated *C. murale* seedlings (Fig. 1*C*), being more intense in untreated ones. Results obtained both by isoelectric focusing and spectophotometrically, argue in favour of correlation between POD isoenzymes and activity with early seedling development, which was delayed in $GA_3$ treated seeds.

These data indicate that POD are not involved in seed germination, while they play a significant role in early *C. murale* seedling development.

In all samples protein concentration was on the same level until 72 h of imbibition (Table 1). A significant increase of protein concentration occurred after 3rd day of imbibition in both untreated and $GA_3$ treated seeds (Table 1), being more expressive in untreated ones. The increase in protein concentration during seed germination is connected with release from storage and *de novo* synthesis of building and regulatory proteins in the emerging seedlings (Roberts 1972). As well as CAT and SOD activity, protein concentration of $GA_3$ treated seeds on the 6th day of germination was on the same level as protein concentration of untreated seeds on the 4th day (Table 1).

We showed that $GA_3$ delayed and synchronized *C.murale* germination. Our results indicate a sequential expression of the antioxidative enzymes and their importance in seed germination. Changes in CAT, SOD and POD activities could be related to different phases of development. Decrease in SOD activity ($H_2O_2$ producing enzyme), increase in CAT and appearance of POD activity ($H_2O_2$ consuming enzymes) coincide with early seedling development in *C. murale.* These data suggest that decrease in $H_2O_2$ level might be involved in regulation of this process.

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