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# ANTIOXIDATIVE ENZYMES DURING GERMINATION OF TWO LINES OF SERBIAN SPRUCE [*PICEA OMORIKA* (PANČ.) PURKYNĚ]

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*Abstract* – Two lines of *Picea omorika* (Panč.) Purkyně were compared with respect to germination percentage as well as specific activity and isoenzyme pattern of catalase, superoxide dismutase, and peroxidase (POD) during germination. Line A had a higher germination percentage and higher enzyme activities in dry seeds and seedlings compared to line C. Peroxidase activity was not detected in dry seeds, but measured up to 10 U/g and 28 U/g on the 7$^{th}$ day of germination in lines C and A, respectively. The most abundant POD basic isoform in seedlings of both lines (pI 8.2) was not found previously in needles of adult Serbian spruce trees of the same lines.

*Key words*: *Picea omorika* (Panč.) Purkyně, seed germination, catalase, peroxidase, superoxide dismutase

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## INTRODUCTION

Seed germination is a complex process that involves the activation of specific enzymes at the appropriate times and regulation of their activity. It is characterized by imbibition, after which seeds rapidly increase oxygen uptake and oxidative phosphorylation, processes required to meet the high energy cost of germination (T o m m a s i, 2001). Oxidative phosphorylation and mobilization of food storage generate reactive oxygen species (ROS) that can cause structural and functional damage in cells. The enzymes responsible for ROS scavenging are therefore of particular importance for the success of germination. Also it has been shown that seed germination percentage might be related to the efficiency of free radical scavenging in dry seeds because this scavenging can affect merely seed storage and vigor (P r i e s t l e y, 1986; B a i l l y et al., 1998). Some authors have shown that production of ROS during seed germination may be a beneficial biological reaction, one that is linked with germination capacity, seedling development, and protection against parasitic organisms during germination (S c h o p f e r et al., 2001). For these reason there is a growing interest in the functional role of ROS and corresponding scavenging enzymic systems in seed germination (B a i l l y et al., 2001; D u č i ć et al., 2002).

Antioxidative enzymes such as superoxide dismutase (SOD), POD, and catalase (CAT) are considered to be the main protective enzymes engaged in the removal of free radicals and activated oxygen species (B l o k h i n a et al., 2003; D e v i et al., 2005). Catalase and SOD are the most efficient antioxidative enzymes (S c a n d a l i o s, 1993). On the other hand, PODs also have a role in very important physiological processes like control of growth by lignification, cross-linking of pectins and structural proteins in the cell wall, and catabolism of auxins (G a s p a r et al., 1991). Despite the importance of PODs in plant development, their exact relationship to developmental events is often obscured by their extensive polymorphism in a single plant species. It is therefore very important to select POD associated with plant development for purification and further studies (J a c k s o n and R i c a r d o, 1998).

Studies of antioxidative enzymes during germination of coniferous trees are rather rare. One such study

treated enzymes involved in cycling of ascorbic acid and glutathione in *Pinus pinea* seeds during the first stages of germination (T o m m a s i et al., 2001).

*Picea omorika* (Panč.) Purkyně is a Balkan endemic coniferous species and Tertiary relict of the European flora. It is an interesting system for germination studies for two reasons. Serbian spruce is more tolerant to air pollution and drought in comparison with other conifers (G i l m a n and W a t s o n, 1994; K r á l, 2002), and trees of this species grow in a wide edaphic and altitudinal range (300-1700 m). Natural regeneration of *P. omorika,* as a pioneer tree species which predominates as an early recruit of forest succession, occurs exclusively within disturbed and relatively open habitats such as cliffs, forest clearings and vegetation gaps (Č o l i ć, 1957, 1966). On the other hand, Serbian spruce is cultivated throughout Europe as a decorative species due to its elegant shape and pollution resistance (J o v a n o v i ć, 1970).

This work is the first study of the activities and isoenzyme pattern of the antioxidative enzymes CAT, POD, and SOD during germination of *P. omorika* seeds. Our aim was to follow the expression of particular parts of antioxidative systems during the early stages of germination of two genetically different lines and compare them with the activities in the needles of Serbian spruce. We also sought to find out if there is a correlation between activities of these antioxidative enzymes and seed germination in these two lines.

## MATERIAL AND METHODS

### *Plant Material*

Seeds were obtained from 15-year-old Serbian spruce trees grown in a generative seed orchard in Godovik (43° 51' N, 20° 02' E, 400 m), Serbia. The generative seed orchard of Serbian spruce was raised on the basis of results obtained in previous studies of collective and individual variability of continous and discontinuous features of half-sib lines (Š i j a č i ć - N i k o l i ć, 2001). The following lines of Serbian spruce were used in the experiments: A ("borealis") – branching similar to the branching in Norway spruce, broad tree crown; and C ("serbica") - branching characteristics typical of trees in the natural habitat of Serbian spruce, narrow pyramidal crown. The seeds were collected in 2002.

Fifty seeds of Serbian spruce were sown in 10-cm (diameter) Petri dishes on filter paper containing 5 mL of distilled water and germinated at 25°C with photoperiod of 12 h for 7 days. The germination percentage was determined in batches of 50 seeds per sample (one Petri dish), using protrusion of the radicle by more than one millimeter as the criterion. A batch of 50 seeds (one Petri dish) was used for fresh weight (FW) determination of seedlings. All measurements were done in tetraplicate after 4, 5, 6, and 7 days of imbibition.

### *Enzyme Extraction*

Whole germinated seeds and/or seedlings (separated from nongerminated seeds) obtained from one Petri dish were powdered in liquid nitrogen. Frozen powder was added to 1.5 mL of extraction buffer containing 100 mM Tris (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 % Triton X-100, 1 mM dithiothreitol (DTT), and 2 % polyvinylpyrolidone (PVP). The suspension was incubated at 4°C for 1 h and than centrifuged for 10 min at 10000 *g* and 4°C. The supernatant was used for POD, CAT, and SOD activity and protein concentration measurements.

### *Enzyme Assays*

Activity of SOD was determined spectrophotometrically at 550 nm in 50 mM sodium phosphate buffer at pH 7.8 with 1 mM EDTA and 0.02 mM sodium azide by measuring the percent of SOD-induced inhibition of cytochrome c reduction using a xanthine/xanthine oxidase system as the source of $O_2^{\cdot-}$ (M c C o r d and F r i d o v i c h, 1968). One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition of cytochrome c reduction.

Catalase activity was determined spectrophotometrically at 240 nm by measuring decrease in absorbance of $H_2O_2$ from 0.850 nm to 0.750 nm in 3 mL of 100 mM sodium phosphate buffer (pH 7.5) at 25°C (B e r g m e y e r, 1983). The extinction coefficient for $H_2O_2$ was 4.32 $cm^2/\mu mol$.

Peroxidase activity was determined spectrophotometrically with guaiacol as the substrate [(modified method of C h a n c e and M a e h l y (1956)]. The assay mixture contained 50 mM sodium acetate buffer (pH 5.5), 92 mM guaiacol, and 18 mM $H_2O_2$ at 25°C. The reaction was monitored at 470 nm and the reaction rate calculated from a coefficient of absorbance for tetraguaiacol

of 25.5 $cm^2/\mu mol$.

One unit of CAT and POD activity was defined as the amount of enzyme that converts one micromole of substrate to product in one minute.

Enzyme activities were referred to the sample fresh weight.

*Native Polyacrylamide Gel Electrophoresis*

Polyacrylamide gel electrophoresis was carried out on the Mini Hoefer SE electrophoresis system under non/denaturing conditions in gels containing 8% polyacrylamide with a 4% stacking gel. A constant current of 25 mA per gel was applied. Electrophoresis buffers and gels were prepared by the method of L a e m m l i (1970) except that SDS was excluded. Equal volumes of all samples were loaded on the gels.

*Isoelectric Focusing*

Isoelectric focusing was performed horizontally in the LKB 2117 Multiphor II system using 1 mm thick polyacrylamide gels (5% T, 3% C) containing 4% 3.5-10.0 ampholites. Gels were solidified with 50 µL of 10% ammonium persulfate and 7 µL of N,N,N',N'-tetramethylethylenediamine (TEMED) per 15 mL of gel solution. Gels were run at 4°C with constant power of 0.5 W/cm of gel width and with limiting voltage of 2000 V for 2 h.

*Enzyme Activity Staining*

Catalase was stained on the gel by incubation in the dark for 20 min in 10 mM $H_2O_2$ dissolved in sodium acetate buffer (pH 5.5), followed by incubation in a mixture of 1 % $K_3Fe(CN)_6$ and $FeCl_3$ for 15 min (W o o d b u r y et al., 1971).

Isoenzymes of SOD were detected on the gels by the method of B e a u c h a m p and F r i d o v i c h (1971). Briefly, the gels were incubated for 20 min in the dark in 20 mL of 100 mM sodium phosphate buffer (pH 7.8) with 4 mg of nitrobluetetrazolium, 0.6 mg of riboflavin, 2 µL of TEMED and 40 µL of 0.25 M NaEDTA. The gels were then briefly rinsed with distilled water and illuminated for 15 min.

Peroxidase was stained on the gel with 9.2 mM guaiacol and 5 mM $H_2O_2$ in sodium acetate buffer (pH 5.5) for 10 min at 25°C (L a g r i m i n i and R o t h s t e i n, 1987).

## RESULTS

*Percentage of Germination*

The percentage of germination was determined for two lines of *P. omorika* named "line A" and "line C" over a period of 7 days. Radicle protrusion occurred on the 2nd day, but the germination percentage increased significantly on the 4th day of germination (see Fig. 1).

The germination percentage of line A was 1.5 times higher than that of line C on the 4th day, 68% and 45%,

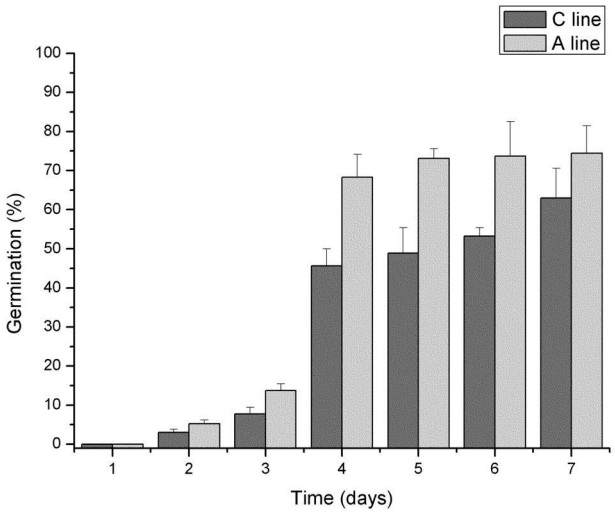


Fig. 1. Seed germination in two different lines of Serbian spruce.

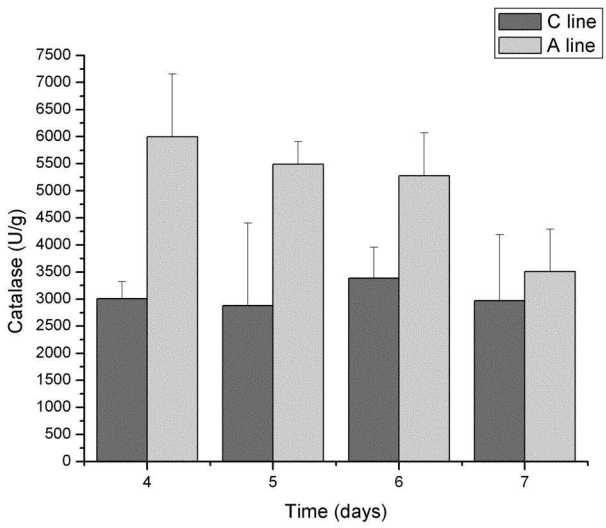


Fig. 2. Specific catalase activity per fresh weight of germinated seeds/seedlings in two different lines of Serbian spruce during germination.

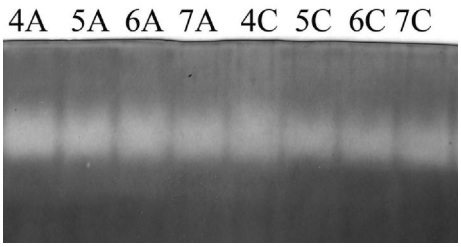


Fig. 3. Isoenzyme pattern of catalase during germination on polyacrylamide gels after native electrophoresis. 4A - 4th day, 5A -5th day, 6A - 6th day, 7A -7th day of germination of line A. 4C - 4th day, 5C - 5th day, 6C - 6th day, 7C - 7th day of germination of line C.

respectively. After the 4th day, the germination percentage increased slightly and this increase was more pronounced for line A. Germination of line C seeds was delayed compared to those of line A. Seven days after the start of imbibition, the germination percentages for both lines were similar: 74% for line A and 63% for line C.

In the next experiments, we measured specific activity of the enzymes catalase, superoxide dismutase, and peroxidase per fresh weight of seedlings from the 4th day, when most of the seeds germinated.

*Enzyme Activities and Isoenzyme Pattern*

As no changes in enzyme activity were detected in *P. omorika* seeds up to 4th day after the start of imbibition, we here present specific activity of the enzymes catalase, superoxide dismutase, and peroxidase from the 4th day, when most of the seeds germinated.

*Catalase*

Catalase activity did not change significantly during germination and was very similar to the catalase activity in seeds before germination (Fig. 2). Specific catalase activity was higher in germinated seeds/seedlings of line A compared to line C. This activity was also higher in dry seeds of line A (4100 U/g) compared to dry seeds of line C (2500 U/g). There was no catalase activity in nongerminated seeds obtained after separation from germinated seeds on Petri dishes after the 7th day of germination.

The catalase isoenzyme pattern did not change during germination, only two close bands of catalase activity being detected on native polyacrylamide gels in all samples (Fig. 3).

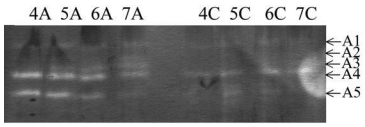


Fig. 5. Activity of SOD on polyacrylamide gels after isoelectrofocusing and native electrophoresis. 4A - 4th day, 5A - 5th day, 6A - 6th day, 7A - 7th day of germination of line A. 4C - 4th day, 5C - 5th day, 6C - 6th day, 7C - 7th day of germination of line C.

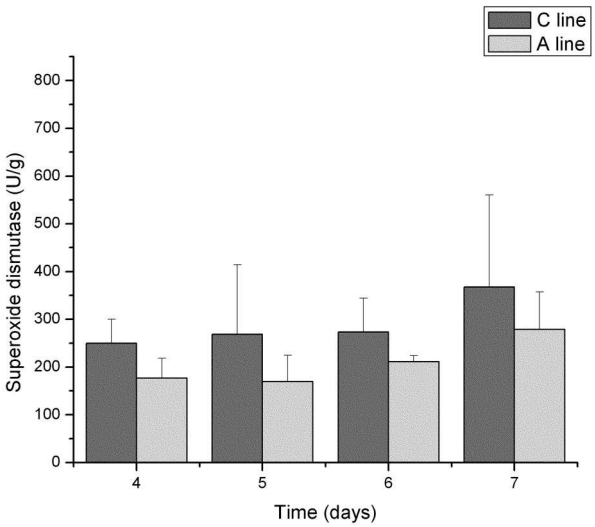


Fig. 4. Specific superoxide dismutase activity per fresh weight of germinated seeds/seedlings in two different lines of *P. omorika* during germination.

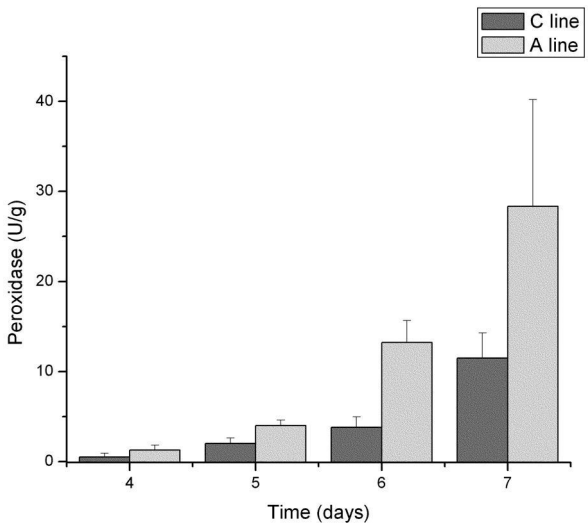


Fig. 6. Specific peroxidase activity per fresh weight of germinated seeds/seedlings in two different lines of Serbian spruce during germination.

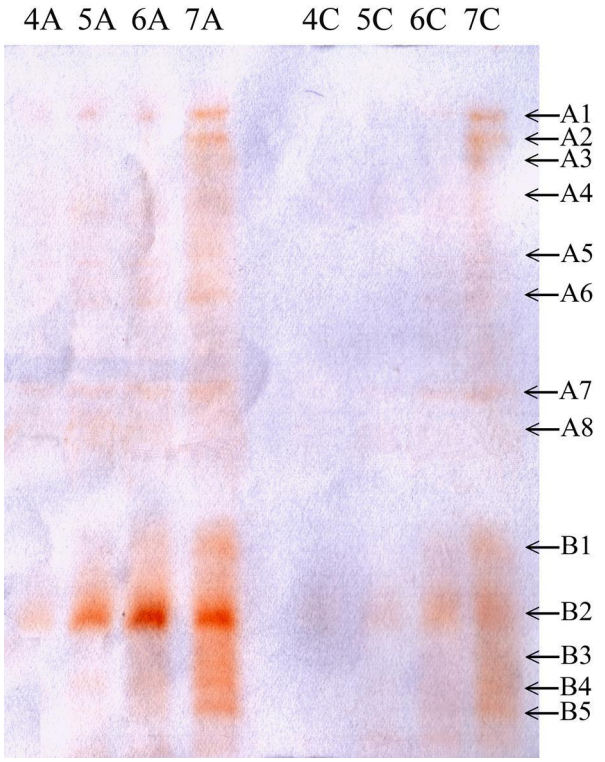


Fig. 7. Isoelectrofocusing of POD isoenzymes from germinated seeds/seedlings of Serbian spruce during germination. 4A - 4th day, 5A - 5th day, 6A - 6th day, 7A - 7th day of germination of line A. 4C - 4th day, 5C - 5th day, 6C - 6th day, 7C - 7th day of germination of line C.

*Superoxide Dismutase*

Superoxide dismutase activity did not change significantly in seedlings during germination (Fig. 4). We obtained similar activities of SOD in dry seeds of both lines (220 ± 50 U/g for line C and 200 U/g ± 40 for line A).

After isoelectrofocusing, five different bands were present in all samples during germination (Fig. 5). All five bands were in the acidic region of the gel with pI values ranging from 4.0 to 4.9 (see Table 1).

All samples had all of the five bands and there was little difference in relative abundance of these five SOD isoenzymes between lines A and C. There was an increase in activity of the A2 and A3 SOD isoforms during germination of line A on the 7th day of germination. The isoenzyme pattern of SOD in line C did not change during germination.

Table 1. pI values of detected isoforms of SOD on isoelectrofocusing polyacrylamide gels.

| Isoform | A1 | A2 | A3 | A4 | A5 |
|---|---|---|---|---|---|
| pI | 4.0 | 4.3 | 4.4 | 4.6 | 4.9 |

*Peroxidase*

Contrary to CAT and SOD activity, POD activity showed significant changes during germination with respect to both specific activity and isoenzyme pattern. In dry seeds and on the first 3 days of germination, there was no POD activity. Specific activity of POD per fresh weight increased continuously from the 4th day and was highest on the 7th day of germination (Fig. 6).

Comparing lines A and C, we see that POD specific activity per fresh weight of germinated seeds/seedlings was almost three times higher for line A than for line C. For example, on the 7th day of germination specific activity of POD in line A was 28 U/g, while for line C it was 10 U/g.

The POD isoenzyme pattern changed, quantitatively and qualitatively, during germination in both lines, the izoenzyme with pI 8.2 being the dominant basic form (Fig. 7).

We detected eight acidic isoforms of POD with pI between 3.2 and 6.5 and five basic isoforms of POD with pI values from 7.5 to 9.1 (Table 2).

The most prominent POD isoform was the basic isoform B2 with pI value of 8.2 (Fig. 7). This isoform was not previously detected in needles of different lines of adult Serbian spruce trees (Bogdanović et al., 2005). Nor was this isoform detected in the needles of Serbian spruce during seasonal changes of the POD isoenzyme pattern (Bogdanović et al., 2007). These

Table 2. pI values of detected isoforms of POD on isoelectrofocusing polyacrylamide gels.

| Isoform | A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | B1 | B2 | B3 | B4 | B5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pI | 3.2 | 3.5 | 3.7 | 4.1 | 4.6 | 5.0 | 5.9 | 6.5 | 7.5 | 8.2 | 8.6 | 8.8 | 9.1 |

results suggest that the basic isoform B2 may be narrowly specific for Serbian spruce seedlings.

## DISCUSSION

We have shown a difference in seed germination between two lines of *P. omorika*, line A being more potent than line C. It is evident that higher CAT activity is present in both germinated seeds/seedlings and dry seeds of line A in comparison with line C. Catalase activity was not detected in non-germinated seeds after 7 days of imbibition. This observation suggests that CAT activity in seeds and seedlings may be involved in preservation of viability during storage and also necessary for seed germination and early seedling growth. This is in accordance with previous results indicating that activity of antioxidative enzymes such as catalase is closely related with storage longevity and germination percentage of bitter gourd seeds (Y e h et al., 2005).

Activity of SOD did not change during germination, was on a similar level in the seeds of both lines, and was also present in dry seeds. It can be concluded that SOD activity is not correlated with differences in seed germination between the two lines. However, its presence in all samples suggests that this enzyme may participate in protection against free superoxide radicals.

Peroxidase activity showed the most notable changes during germination. Dry seeds exhibited showed no POD activity, but during germination this activity appeared and dramatically increased. From this fact it could be concluded that POD activity may have a role in the later stages of germination and in seedling development, but not in preservation of dry seeds. Peroxidase activity was not detected even in imbibed seeds before the start of germination in tomato (M o r o h a s h i, 2002) and *Chenopodium rubrum* (D u č i ć et al., 2003/4; M i t r o v i ć et al., 2005).

The observation that line A shows both a higher seed germination percentage and higher specific POD activity in germinated seeds/seedlings compared to line C points to possible involvement of POD in seedling development. This may involve a higher rate of metabolic processes in seedlings of line A compared to line C. Peroxidase activity has also been shown to increase during late germination and early seedling growth in some herbaceous species: the annuals *C. rubrum* (D u č i ć et al., 2003/4) and tomato (M o r o h a s h i, 2002); the biannual *Brassica oleracea* (B e l a n i et al., 2002); and the perennial *Viola carnuta* (M i t c h e l l and B a r r e t t, 2000).

The basic POD with pI value of 8.2 was the most abundant isoform in seedlings and was not found in needles of adult Serbian spruce trees during annual changes of isoenzymes (B o g d a n o v i ć et al., 2007). This fact suggests that the given basic isoform has a special role only in germination and early stages of seedling development. These results are in agreement with certain findings of J a c k s o n and R i c a r d o (1998), which showed that basic isoforms of POD are clearly linked with vegetative development of lupin and important role in the early stages of lupin growth. Therefore, the purification and characterization of this basic POD isoform with pI value of 8.2 could be very important for understanding the precise role of POD in early stages of *P. omorika* development.

In summary, we can conclude that activities of the antioxidative enzymes CAT and SOD may be involved in preserving the viability of seeds and protecting them from reactive oxygen species formed during storage and seed germination. Catalase activity in seeds can serve as a parameter that indicates the germination capacity of dry seeds. Peroxidase activity may have a role in the early stages of development of Serbian spruce seedlings. The most important POD isoform for seedling growth is the basic one with pI value of 8.2. In order to further investigate the role of POD in the early stages of seedling development, this isoform of POD will be analyzed by isolation and kinetic characterization.

It can be concluded that the higher activity of CAT and POD in line A in comparison with line C is a parameter that indicates higher seed viability.

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# ЕНЗИМИ ЗАШТИТЕ ОД ОКСИДАЦИОНИХ ОШТЕЋЕЊА У ТОКУ КЛИЈАЊА ДВЕ ЛИНИЈЕ ОМОРИКЕ [PICEA OMORIKA (PANČ.) PURKYNĚ]

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Поређени су проценат клијања, специфична активност и изоензимски профил каталазе, супероксид дисмутазе и пероксидазе у две линије оморике [*Picea omorika* (Panč.) Purkyně] у току клијања. Линија А је имала већи проценат клијања и веће ензимске активности у сувом семену и клијанцима, у поређењу са линијом С. Пероксидазна активност није нађена у сувим семенима, а повећавала се до 10 U/g и 28 U/g седмог дана клијања у линијама С и А, респективно. Најзаступљенија базна изоформа пероксидазе у клијанцима обе линије (pI 8.2) није нађена раније у четинама одраслих јединки истих линија.