Activities of amine oxidase, peroxidase and catalase in seedlings of *Pisum sativum* L. under different light conditions

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ABSTRACT

The activities of amine oxidase, peroxidase and catalase were studied in 12 cultivars of field pea (*Pisum sativum* L.) and one accession of wild pea (*Pisum sativum* subsp. *transcaucasicum*). The influence of different light conditions on the enzyme activities was studied in extracts of 8-d-old seedlings. Substantially higher amine oxidase activity was detected in etiolated pea seedlings than in plants growing under controlled light conditions (12h photoperiod). Higher peroxidase and catalase activities indicate more intensive production of toxic hydrogen peroxide evolved by reactions of different type in green plants in comparison with etiolated ones. Significantly lower activity of peroxidases in etiolated plants could be related to a lower degree of lignification. Marked differences in enzyme activities between etiolated field pea and *P. sativum* subsp. *transcaucasicum* were observed for all studied enzymes. A very interesting result was the exceptionally low activity of amine oxidase in etiolated plants that was hardly detectable in green plants of Malton cultivar. Two bands with amine oxidase activity were found by the method of native PAGE in extracts of 8-d-old plants. A different relationship of these isoenzymes was detected in field pea and wild pea. Two isoenzymes were present in pea shoots but only one isoenzyme was detected in pea roots. Amine oxidase isoenzymes were studied in the roots and shoots of cv. Smaragd for three weeks. The profile of isoenzymes was opposite in 8- and 36-d-old stems of pea.

Keywords: amine oxidase; peroxidase; catalase; pea; isoenzymes; lignification

Copper-containing amine oxidase (EC 1.4.3.6) [amine:O_2 oxidoreductase (deaminating)] catalyzes oxidative deamination of biogenic amines to the respective aldehydes and ammonia, accompanied by two-electron reduction of molecular oxygen to hydrogen peroxide (Bachrach 1985):

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$$

The enzymes with this activity were identified and described especially in leguminous plants (Luhová et al. 1998, Medda et al. 1995). The profile of amine oxidase activity was determined in different organs of dark-grown lentil seedlings while epicotyls showed the highest level of enzyme activity. Two isoenzymes of lentil amine oxidase having different isoelectric points were found in lentil seedlings (Maccarrone et al. 1996). The activity of amine oxidase is high in the middle of the lamellar region (Federico and Angelini 1986, Liu et al. 1995). Immunolocalization studies demonstrated the presence of amine oxidase in cortical cell walls and in xylem tissue (Angelini et al. 1990). It was suggested that H_2O_2, generated by amine oxidation, was important for lignification both in normal and stress conditions (Angelini et al. 1993, Rea et al. 1998).

Plant peroxidases (EC 1.11.1.7) [donor:hydrogen peroxide oxidoreductase] are monomeric heme-containing enzymes that are usually glycosylated and that catalyze a large variety of reactions (Siegel 1993):

$$donor + H_2O_2 \rightarrow oxidized\ donor + 2H_2O$$

Isoperoxidases, arising from the transcription of different genes or from post-translational modification, are widely distributed within both the intra- and extracellular environment (Jackson and Ricardo 1994). Peroxidases have been studied for their important role in lignification and suberization, for their active participation in the formation of diphenyl bridges, cross-linking of hydroxyproline-rich proteins (extensin) in the cell wall matrix and for their control function of redox state in the apoplast. The involvement of peroxidases in stress-related physiological processes (Low and Merida 1996) as well as in plant-pathogen interactions was demonstrated (Montalbini et al. 1995, Wojtaszek 1997).

One of the main enzymes playing a role in the catabolism of hydrogen peroxide is catalase (EC 1.11.1.6) [hydrogen-peroxide:hydrogen-peroxide oxidoreductase] (Chance et al. 1979). The catalase is a tetrameric heme protein, occurring in almost all aerobic organisms. This enzyme is one of the few enzymes that exhibit dual enzyme activity. It has hyperoxidase activity (catalytic activity) when catalyzes the dismutation of hydrogen peroxide into water and oxygen:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The other catalase activity is peroxidase activity (peroxidative activity) when the substrates are one molecule of hydrogen peroxide and one molecule of hydrogen donor:

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donor + H_2O_2 → oxidized donor + $2H_2O$

The enzyme with catalase activity is present as multiple isoforms in plants and recent research on catalase cDNA clones showed that catalases exist as small gene families (Scandalios 1994). Plant catalases are predominantly peroxisomal enzymes and most of them contain a carboxy terminal consensus sequence for peroxisomal import (Gould et al. 1988). Catalases play a role of specific peroxidase and their function is to protect cells from toxic effects of hydrogen peroxide.

Selected enzymes play an important role in defence responses of plants to biotic or abiotic stress (Lebeda et al. 1999, 2001, Luhová et al. 1999). Their isoenzyme patterns are intensively studied for cultivar identification in breeding, seed marketing and in the other fields of agriculture (Samec et al. 1998). The main task of the present study was to examine and to explain reciprocal relations between the enzyme reactions participating in metabolism of hydrogen peroxide in pea grown under extreme (dark) and controlled light conditions.

MATERIAL AND METHODS

Plant material

The seeds of field pea (*Pisum sativum* L.) cultivars Lantra, Komet, Olivín, Dětenický žlutý, Bohatýr, Malton, Smaragd, Milion zelený, Carrera, DP 1059, Adept and Janus were obtained from Agritec Ltd. (Šumperk, Czech Republic) and the seeds of wild *Pisum sativum* subsp. *transcaucasicum* came from Nordic Gene Bank (Lund, Sweden). The seeds were soaked in distilled water for 24 h, transferred onto a Perlite EP AGRO (Perlit, Šenov near Nový Jičín, Czech Republic) layer, irrigated with tap water. The plants were cultivated at the temperature 18°C, either in dark or at 12h photoperiod and light intensity 100 µmol/m/s over day, and 15°C over night in a temperature controlled growth chamber.

Preparation of plant extracts

Plant material (from 8- to 36-d-old seedlings) was homogenized at a ratio of 1:2 (w/v) with 0.1M potassium phosphate buffer, pH 7.0. Extracts were filtered through nylon cloth and centrifuged at 12 000 × g, for 30 min at 4°C.

Enzyme activity assays

Enzyme activities were assayed by spectrophotometric methods. The amine oxidase activity was determined using a coupled reaction with horseradish peroxidase and guaiacol (Frébort et al. 1989). The reaction mixture (final volume: 1.7 ml) contained 0.1M potassium phosphate buffer, pH 7.0, 0.5mM guaiacol, 10 nkat of peroxidase and plant extract. The reaction was started by injecting 50 µl of putrescine (final concentration: 2.5mM), and a time-dependent increase in absorption at 436 nm (ε – 4500/M/cm) and at 30°C was recorded for a period of 3 min.

Peroxidase activity was measured by a modified method with guaiacol (Angelini et al. 1990). The reaction mixture (final volume: 1.75 ml) contained 0.1M potassium phosphate buffer, pH 7.0, and 5mM guaiacol and plant extract. The absorbance at 436 nm (ε – 4500/M/cm) was continuously detected for 1 min at 30°C after adding 0.2 ml of 1M hydrogen peroxide.

Catalase activity was measured by an assay of hydrogen peroxide based on formation of its stable complex with ammonium molybdate (Góth 1991). 0.2 ml of plant extract was incubated in 1 ml reaction mixture containing 65mM hydrogen peroxide in 60mM sodium-potassium phosphate buffer, pH 7.4 at 25°C for 4 min. The enzymatic reaction was stopped with 1 ml of 32.4mM ammonium molybdate and the concentration of the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm.

Protein concentration was determined according to Bradford with bovine serum albumin as a standard (Bradford 1976).

Measurements were determined in two independent experiments at least, each performed with three replications.

Native PAGE

Electrophoretic separations were performed on non-denaturing PAGE using 4.5% stacking gel and 10% separation gel with 0.025M Tris – 0.19M glycine buffer, pH 8.3, at 4°C. Detection of amine oxidase activity was performed in 12.5 ml of 0.1M K-phosphate buffer, pH 7.0, containing 15 mg of putrescine and 1.5 mg of horseradish peroxidase after addition of 7.5 mg of 4-chloro-1-naphthol to 2.5 ml of cold methanol (Houen and Leonardsen 1992).

Western immunoblotting

Western immunoblotting was performed according to Wilson and Nakane (1978). Amine oxidase was separated on native gels and electroblotted to the Immobilon polyvinylidene difluoride membrane (Sigma). After 2h blocking of free binding sites onto the membrane with 3% gelatine in Tris/NaCl (20mM Tris, 500mM NaCl, pH 7.5) and washing with Tris/NaCl/Tween 20 (20mM Tris, 500mM NaCl, 0.05% Tween 20, pH 7.5), the membrane was incubated for 2 h with rabbit anti-AO antiserum diluted 1:150 in Tris/NaCl. The non-specifically bound antibodies were removed by washing in Tris/NaCl/Tween, and the membrane was incubated for 2 h with protein A – horseradish peroxidase (3500-fold diluted) in Tris/NaCl with 1% gelatine. The coloured peroxidase reaction was carried out using a fresh methanol solution of 4-chloro-1-naphtol (0.5 mg/ml) and 0.1% hydrogen peroxide in Tris/NaCl. The staining reaction was stopped by rinsing the membrane with water.

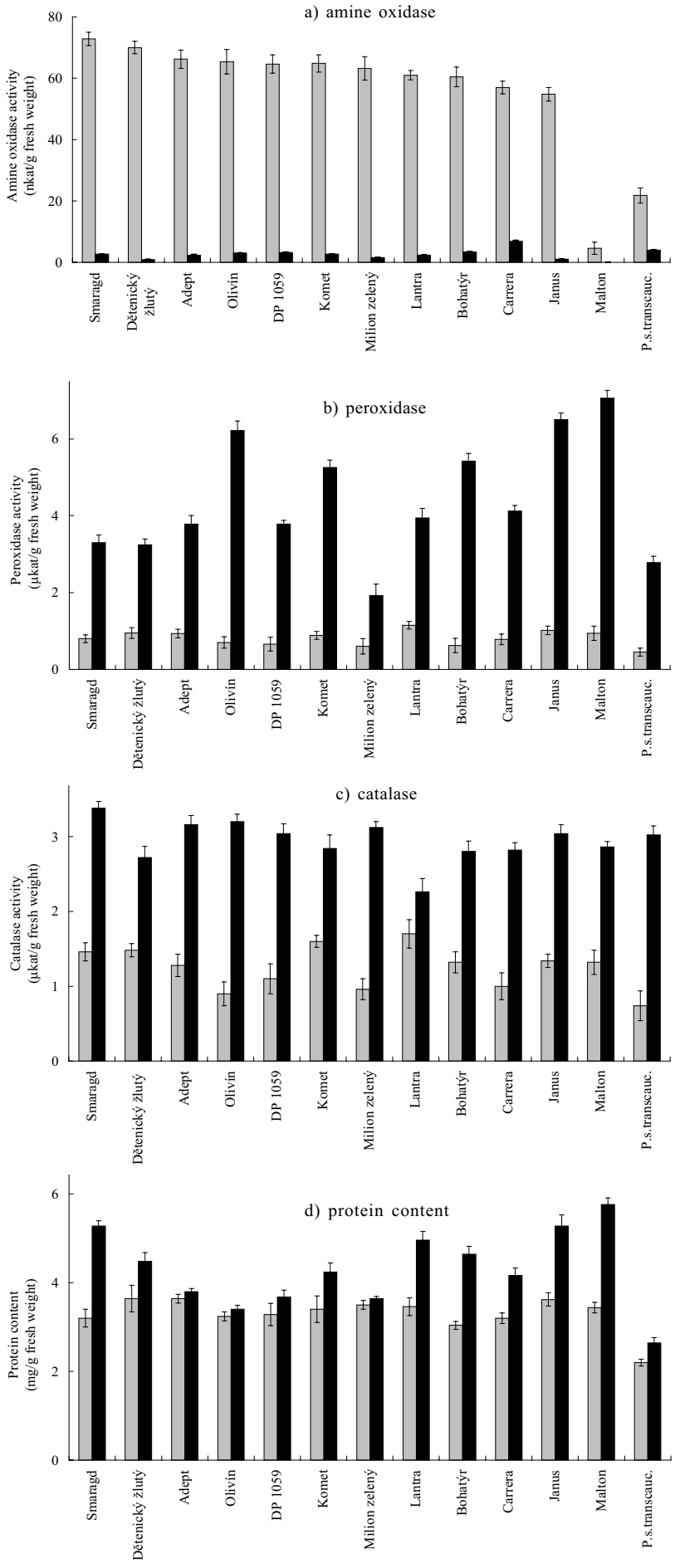

Figure 1. Amine oxidase (a), peroxidase (b), catalase (c) activities and protein content (d) per gram of fresh weight of plant material after extraction with 0.1M potassium phosphate buffer, pH 7.0

Histochemical study

Detection of lignin was performed by phloroglucinol/HCl staining (Bate et al. 1994). The sections of roots and stems were immersed in 10% phloroglucinol for 2 min followed by immersion in concentrated HCl for 1 min, and then they were washed in 75% glycerol for 5 min. The observation was done on cross-sections (50 µm) using Olympus microscope, model BX50. The cell wall strength was measured by a microscope using a special micrometric gauge on the objective.

RESULTS

Enzyme activities in etiolated plants and in plants grown under 12h photoperiod

The detected amine oxidase activities were 5–80 times higher in etiolated plants than in plants grown under controlled light conditions. The activities in the extracts of 11 cultivars of field pea grown in dark amounted to 54–73 nkat/g of fresh weight (Figure 1a). Cv. Malton with 4.6 nkat/g of fresh weight in etiolated plants and with undetectable activity in green plants was the only exception. Peroxidase activities were 3–9 times and catalase activities 1–3 times lower in etiolated plants than in plants grown under 12h photoperiod (Figure 1b, c). Higher protein content was typical of green plants (Figure 1d).

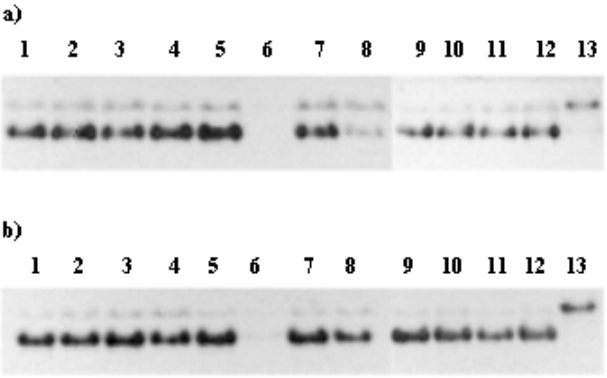

Figure 2. Isoenzyme patterns of amine oxidase activity identified by native PAGE in extracts of plants grown under 12h photoperiod (a) and of etiolated plants (b); lane: 1 – Lantra, 2 – Komet, 3 – Olivín, 4 – Dětenický žlutý, 5 – Bohatýr, 6 – Malton, 7 – Smaragd, 8 – Milion zelený, 9 – Carrera, 10 – DP 1059, 11 – Adept, 12 – Janus, 13 – *P. sativum* subsp. *transcaucasicum*

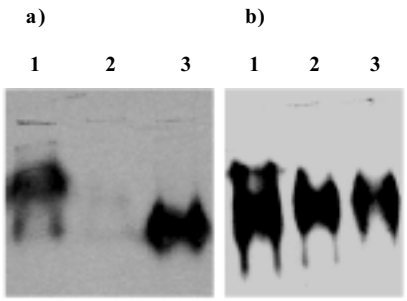

Figure 3. Detection of amine oxidase activity in extracts of *P. sativum* subsp. *transcaucasicum* (1), cvs. Malton (2) and Olivín (3) by native electrophoresis (a) and Western blot following native electrophoresis (b)

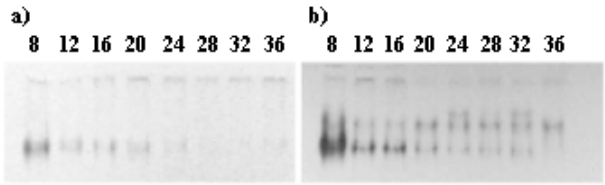

Figure 4. Amine oxidase activities detected by native PAGE in extracts of roots (a), stems and leaves (b) of *P. sativum*, cv. Smaragd, during development of plants under 12h photoperiod; the plants were 8- to 36-d-old

Comparison of enzyme activities in cultivars and wild accession of pea

The amine oxidase activity (22 nkat/g of fresh weight) in etiolated *P. sativum* subsp. *transcaucasicum* was markedly lower than in cultivars of pea grown in the dark (Figure 1a). There also existed a difference in enzyme activities between etiolated pea and wild accession for peroxidase and catalase activities (Figure 1b, c). No such relationship was observed in green plants.

Electrophoretic patterns of amine oxidase

Two bands with amine oxidase activity were detected on gels by native PAGE in the extracts of etiolated and green plants. The isoenzyme with higher mobility was more intensive in all cultivars of pea except *P. sativum* subsp. *transcaucasicum* (Figure 2). Only cv. Malton (Figure 2, lane 6) did not show any visible bands with amine oxidase activity. The Western blot method was used for the study of amine oxidase in cv. Malton and in wild pea. A black band with the same intensity was detected in extracts of cvs. Olivín, Malton and *P. sativum* subsp. *transcaucasicum* after immunostaining (Figure 3b). The relation of the intensity of bands after native PAGE and immunoblotting was opposite for *P. sativum* subsp. *transcaucasicum* (Figure 3a, b). Development of amine oxidase isoenzymes was studied in the roots and shoots of cv. Smaragd for three weeks. The amine oxidase activity decreased in plants older than 8 days. The profile of the isoenzyme was opposite in 8- and 36-d-old stems of pea. Only the isoenzyme with higher mobility was found in roots (Figure 4).

Table 1. Lignification of cell wall; the width of lignified cell wall in the roots and stems of plants grown under 12h photoperiod and of etiolated ones; measurements were performed in 8-d-old plants

	Width of lignified cell wall (μm)	
	root	stem
Etiolated plants	2.5 ± 0.5	2.4 ± 0.4
Green plants	4.2 ± 0.5	3.0 ± 0.5

Lignification in plants grown under different light conditions

Higher content of lignin deposition was confirmed in cv. Smaragd grown under 12h photoperiod. The width of lignified cell wall in the roots and stems of pea grown under 12h photoperiod was larger in comparison with etiolated plants (Table 1).

DISCUSSION

The results confirm that the amine oxidase activities in etiolated pea are several times higher than in plants grown under controlled light conditions (Macholán and Minář 1974). This finding was reported for different *Fabaceae* plants (Federico and Angelini 1988, Maccarrone et al. 1991, Medda et al. 1995). The relationship between amine oxidase, catalase and peroxidase activities can be very interesting for the explanation of reciprocal physiological and biochemical effects of these enzymes in plants germinating under contrasting light conditions. Higher peroxidase activities can be related with higher degree of lignification in green plants in comparison with etiolated ones (Figure 1b, Table 1). The relationship between lignin deposition and the enzymes with amine oxidase and peroxidase activities was shown in light grown chick-pea stems (Angelini et al. 1990). Participation of these enzymes in the lignification process is quite obvious from many studies on the influence of biotic or abiotic stress factors on plants, e.g. wounding, attack of pathogen (Angelini et al. 1993, Rea et al. 1998). The markedly higher amine oxidase activity of etiolated pea correlates with faster growth requiring e.g. faster metabolism of polyamines (Raina and Janne 1975). More intensive production of toxic hydrogen peroxide could be expected on the basis of higher peroxidase and catalase activity in light grown seedlings. The amine oxidase mechanism in green plants, in comparison with etiolated ones, cannot be considered as one of the main sources of hydrogen peroxide production.

Substantial differences in enzyme activities between etiolated pea and accession of wild pea were observed mainly for the activity of amine oxidase that was twice lower in *P. sativum* subsp. *transcaucasicum* in comparison with cultivars of *P. sativum*. However, no such relationship was found in green plants. The levels of enzyme activity studied in pea extracts do not appear to be significantly characteristic to enable a description of the plant type.

High amine oxidase activity is typical of *Fabaceae* plants, especially of *P. sativum*. The markedly low activity of this enzyme in etiolated plants and no activity in green plants of cv. Malton could be considered as a very interesting result. The determination was performed in three samples of cv. Malton originating from various sources and the activity amounted to maximally 4.6 nkat/g of fresh weight in etiolated plants. However, comparable presence of protein belonging to amine oxidase was detected by the method of Western blot in extracts of cvs. Malton and Olivín. The existence of some natural inhibitors in cv. Malton can be an explanation of this anomaly. Natural inhibitors of amine oxidase were reported in *Arachis hypogea* (Sindhu and Desai 1980), *Sorghum vulgare* (Joshi and Prakash 1982) and *Phaseolus vulgaris* (Cogoni et al. 1989). The inhibition effect of an extract from cv. Malton on amine oxidase isolated from cv. Smaragd was observed in previous experiments (data not reported). A possible change on the level of gene, e.g. mutation, post-translational modification or translation activity, can be another explanation of the low activity of amine oxidase in cv. Malton.

Two bands with amine oxidase activity were found by native PAGE in the extracts of whole 8-d-old plants (Figure 2). The quickly moving band was more intensive in all pea cultivars under study. The extract of *P. sativum* subsp. *transcaucasicum* was the only exception containing a significantly more active slowly moving isoenzyme. However, the intensity of the more quickly moving isoenzyme band, detected by the method of Western blot, was comparable with pea cv. Olivín (Figure 3b). The isoenzyme differences were detected between the roots and shoots of pea. The presence of two isoenzymes was confirmed in the extracts of pea shoots, and only one isoenzyme was found in root extracts. Its mobility corresponded with quickly moving isoenzyme from the shoots (Figure 4). The intensity of slowly moving isoenzyme in shoots increased during the development of cv. Smaragd (Figure 4b). Localization of amine oxidase isoenzymes and their function during development of plants are interesting questions for further research on amine oxidase in pea. Currently, the presence of at least two isoenzymes with different molecular properties and different localization in plant organs was identified only in lentil (*Lens culinaris*) seedlings and these isoenzymes differed also in their responsiveness to specific stress factors (Maccarrone et al. 1996).

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ABSTRAKT

Aktivita aminoxidázy, peroxidázy a katalázy v semenáčcích hrachu setého *Pisum sativum* L. pěstovaných za rozdílných světelných podmínek

Aminoxidázová, peroxidázová a katalázová aktivita byla studována u 12 odrůd hrachu setého (*Pisum sativum* L.) a u plané formy *Pisum sativum* subsp. *transcaucasicum*. V extraktech z osmidenních semenáčků byl sledován vliv světelných podmínek na enzymovou aktivitu. Byla stanovena výrazně vyšší aktivita aminoxidázy v etiolovaných rostlinách v porovnání s rostlinami pěstovanými za kontrolovaných světelných podmínek (12h fotoperioda). Vyšší peroxidázová a katalázová aktivita poukazuje na mnohem intenzivnější produkci toxického peroxidu vodíku v zelených rostlinách. Nizká peroxidázová aktivita v etiolovaných rostlinách by mohla souviset s nižším stanoveným stupněm lignifikace. Výrazný rozdíl v aktivitách studovaných enzymů byl pozorován mezi polními odrůdami hrachu setého a planou formou *P. sativum* subsp. *transcaucasicum*. Zajímavým výsledkem byla rovněž velmi nízká aminoxidázová aktivita u etiolovaných semenáčků a nedetekovatelná aktivita u zelených rostlin odrůdy Malton. Metodou nativní PAGE byly na gelech rozlišeny dva izoenzymy s aminoxidázovou aktivitou v extraktech nadzemní části osmidenních semenáčků hrachu. Byl pozorován rozdíl v zastoupení izoenzymů mezi kulturními hrachy a planou formou hrachu. Izoenzymy s aminoxidázovou aktivitou byly studovány v průběhu tří týdnů v kořenech a nadzemní části odrůdy Smaragd. Profil izoenzymů se lišil u 8 a 36 dnů starých stonků hrachu.

Klíčová slova: aminoxidáza; peroxidáza; kataláza; hrach; izoenzymy; lignifikace

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