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Cytochrome Oxidase and Ascorbic Acid Oxidase Activities in Cereal Plants

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Abstract. Cytochrome oxidase and ascorbic acid oxidase activities were investigated in rye, wheat, barley and oat plants. The variations in the activity of both enzymes was followed in the course of the initial 28 days of growth, as well as at the phase of milk ripeness, namely in the cytoplasmic and mitochondrial cell fractions of roots, leaves and spikes.

Both enzymes were active in all measurements. Cytochrome oxidase mostly exhibited a higher activity than ascorbic acid oxidase. The activity of the former enzyme was substantially higher in the mitochondrial fraction of leaves, roots and spikes of the four experimental plants in comparison with the cytoplasmic fraction. On the contrary, the ascorbic acid oxidase activity varied in both cell fractions according to the plant species, organ and growth phase.

The variations in the activity of both enzymes exhibited on the whole a course similar to that of the respiration rate. During the first 14 to 21 days of growth the enzyme activities increased up to the maximum. This was then followed at first by a rapid, later on by a slow decrease. The course of variations in the enzyme activities was, with certain exceptions, alike in all the four plant species investigated.

The significance of individual oxidative enzymes in the respiratory mechanism of plants has not been fully elucidated as yet. Only cytochrome oxidase was proved to participate in the oxidation chain coupled with phosphorylation processes. With other oxidative enzymes abundantly occurring in plant tissues, as *e.g.* peroxidase, polyphenol oxidase, ascorbic acid oxidase, flavin oxidases and others, the direct linkage with the energetic metabolism could not be demonstrated so far. As pointed out in many papers, these enzymes are responsible for an enhanced, energetically ineffective respiration of plant tissues induced especially by certain pathogens (ALLEN 1954, PETINOV and MALYSHEVA 1960, HANUŠOVÁ 1969, MILLIKAN and SANIEWSKI 1972). The enhancement of oxidative processes under these conditions is considered as a defense response of plant tissues (RUBIN and ARTSIKHOVSKAYA 1960, FARKAS and KIRÁLY 1962). The centre of the processes of oxidative phosphorylation is represented by mitochondria (LEHNINGER 1965). One of the criteria for considering the significance of oxidative enzymes in the respiratory metabolism may therefore be their localization

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within the cell. The present paper deals, on this point, with the study of variations in cytochrome oxidase and ascorbic acid oxidase activities in rye, wheat, barley and oat plants during growth, in both mitochondrial and cytoplasmic cell fractions.

Material and Methods

Plants of Wheat (*Triticum aestivum* L. — cv. 'Kralická zlatka'), rye (*Secale cereale* L. — cv. 'Těšovské'), barley (*Hordeum distichon* L. — cv. 'Denár') and oat (*Avena sativa* L. — cv. 'Diadém') were used for the experiments.

Grains were allowed to germinate in Petri dishes on filter papers moistened with distilled water, in an incubator at 23 °C. After three days they were used for the first analysis. For further cultivation the seedlings were planted out into cultivation vessels of 10 l in volume, viz. 50 plantlets per vessel, and cultured in Richter's nutrient solution supplemented with microelements in the form of Hoagland's A—Z solution. The nutrient solution was replaced every 2 weeks. The cultivation took place in a cultivation room illuminated by fluorescent tubes of 10 000 lx 14 h per day. The temperature of the room was maintained at 23 ± 1 °C with a 45% relative air humidity. At the same time another group of plants was cultivated on small areas in the experimental garden.

For the analysis representative parts of shoots and roots were sampled. For the first determination the whole shoots were taken up, later on dark green intact leaf blades were always employed. The roots of plants cultivated in a water culture were used as a whole. For the determination of enzyme activities at the stage of milk ripeness dark green leaf blades, spikes and the basal part of roots of plants grown in the open air were taken. The tissue extract was obtained by homogenizing 6 g fresh weight of the tissue with 30 ml distilled water in a mortar in a cooled room at 2 °C, and by successive centrifuging at 2 500 *g* for 10 min in a cooled centrifuge. The supernatant was then submitted to further centrifugation in a cooled centrifuge at 20 000 *g* for 30 min. The resulting supernatant served as a cytoplasmic fraction, the sediment, as resuspended in 19 ml distilled water, as a mitochondrial one. Both fractions were used for analysis immediately after isolation.

The activity of both enzymes was determined by the manometric technique in a Warburg's respirometer. The composition of the reaction mixture for the determination of cytochrome oxidase activity was as suggested in the manual by Paech and Tracey (1955) with one modification consisting in using hydroquinone as an electron donor instead of ascorbic acid. This alteration had to be made owing to the presence of active ascorbic acid oxidase in the plants tested. A correction had to be made in oxygen uptake for the autooxidation of hydroquinone which occurred at pH 7.3. For the determination of ascorbic acid oxidase activity the same method as in Plhák's paper (1965) was employed. Along with 3 ml of tissue extract, 1 ml of the respective 0.15 M phosphate buffer was pipetted into the vessels, but the concentration of ascorbic acid was reduced to 10 μM per vessel. At higher concentrations of ascorbic acid the acidity of the reaction mixture increased under the conditions of our experiments. The reduced concentration of the substrate was by no means a limiting factor of the ascorbic acid oxidase activity in the plant material examined. In the actual measurement the activity of ascorbic acid oxidase was tested at pH 5. At this pH no autooxidation of ascorbic acid occurred, so that there was no need to correct the data obtained. The correction was performed when determining the dependence of the enzyme activity on pH, namely at the pH's higher than 5. The enzyme activities were followed in the course of 30-min intervals at 25 °C. They were expressed in terms of O_2 uptake per mg protein of tissue extracts per 30 min.

The content of proteins in tissue extracts was determined spectrophotometrically using Folin reagent according to Lowry *et al.* (1951) without previous purification. Only with samples taken from green plant parts the adverse effect of leaf pigments had to be previously eliminated by shaking the tissue homogenates with ethyl ether till decolourization.

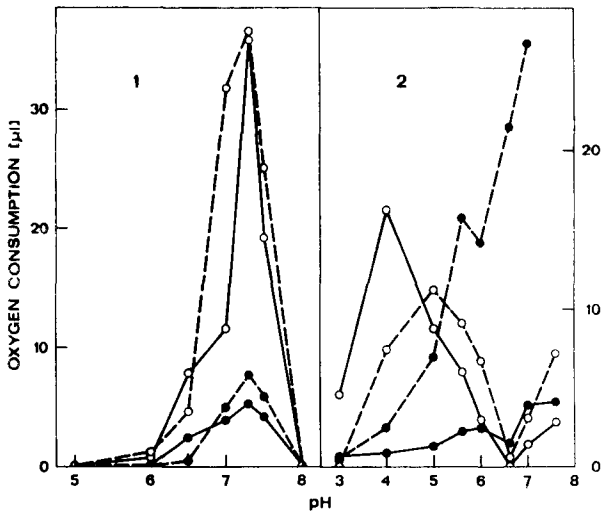
The statistical evaluation of the results was carried out by calculating the standard deviation of mean.

Results

The first part of our experiments aimed at verifying certain reaction conditions for both enzymes given in literature for other biological subjects. For these experiments rye was chosen as a model plant.

The optimum pH value of the reaction medium for the determination of cytochrome oxidase to be 7.3 according to James (1953), was confirmed for

both cell fractions of rye plants (Fig. 1). 67 μM cytochrome *c* per 1 ml reaction solution was found to be sufficient. Optimum pH values of the reaction solution for the determination of ascorbic acid oxidase in the mitochondrial fraction of both roots and shoots of rye seedlings fluctuated in the range of 4—5 (Fig. 2). In the case of the cytoplasmic fraction no expressive optimum



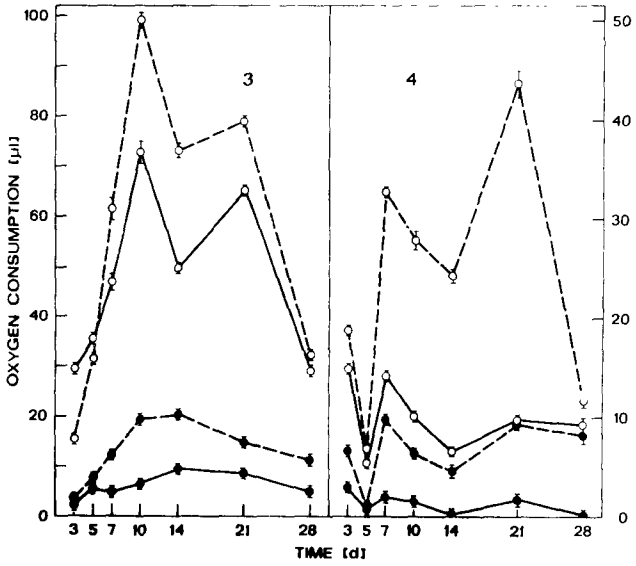
Figs. 1 and 2. The dependence of cytochrome oxidase (1) and ascorbic acid oxidase (2) activities of 3 days old rye seedlings on pH of the reaction medium. The enzyme activity is expressed in μl oxygen uptake per mg protein per 30 min. Mitochondrial (○) and cytoplasmic (●) fractions of shoots (full lines) and roots (dashed lines).

was found within the range of pH 3—8. The appropriate concentration of ascorbic acid amounted to 3.33 μM per 1 ml tissue extract. The course of oxygen uptake for the oxidation of both substrates mentioned was linear within the 30 min interval. The specificity of enzymic oxidation of the substrates mentioned was verified using the inhibitors KCN and DIECA (sodium diethyldithiocarbamate). The inhibition of cytochrome oxidase by a 10^{-3} M solution of cyanide and that of ascorbic acid oxidase by a 10^{-4} M solution of DIECA amounted to almost 100 per cent in both shoots and roots of rye seedlings. Since mitochondria in the reaction medium did not exhibit any measurable oxygen uptake in the presence of substrate, endogenous respiration was not taken into consideration.

The variations in cytochrome oxidase activity in the course of the first 28 days of growth of the plants examined are demonstrated in Figs. 3, 5, 7 and 9. The enzyme was active mostly in both cell fractions of roots and leaves. However, the enzyme activity in the mitochondrial fraction of both organs was substantially higher in comparison with the cytoplasmic one. During a 28-day cultivation the course of variations in cytochrome oxidase activity was very similar with rye, wheat and barley plants. Between the 3rd to 14th day of growth the enzyme activity gradually increased and reached the maximum. Following this period it gradually decreased. In oat plants the initial enzyme activity decreased to the 7th day almost to the minimum,

then it increased up to the 21st day to the level of initial activity. Later on it again decreased.

Cytochrome oxidase was active in all the four plant species even at the stage of milk ripeness. Its activity was predominantly concentrated only in

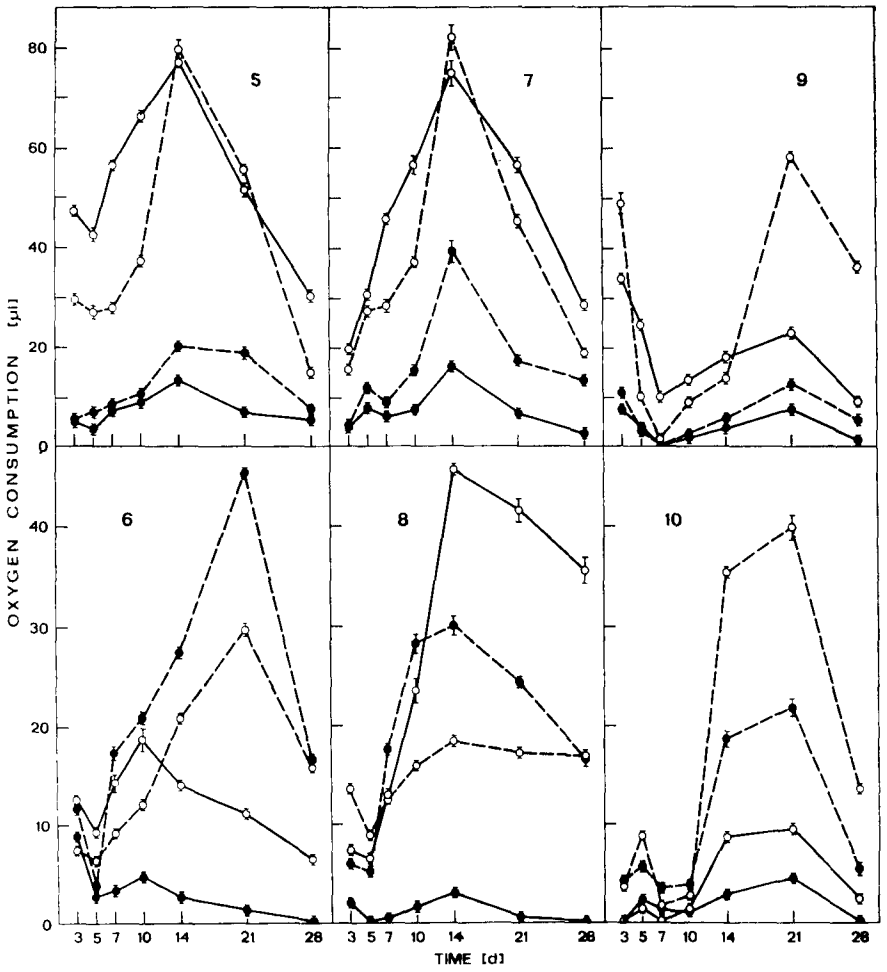


Figs. 3 and 4. The variation in cytochrome oxidase (3) and ascorbic acid oxidase (4) activities of rye plants in the course of a 28-day cultivation under artificial conditions. For further caption see Figs. 1 and 2.

the mitochondrial fraction. The values of its activity were lower than at early growth phases. In roots the activity determined was a little higher than in spikes and leaves.

Ascorbic acid oxidase was active in the leaves and roots of all four plants tested in the course of their 28-day growth, as well (Figs. 4, 6, 8 and 10). Its activity was on the whole approximately by one half lower than that of cytochrome oxidase and its participation in both cell fractions varied according to the species and organ. In rye and oat the enzyme was relatively most active in the mitochondrial fraction of roots. In root cytoplasmic fraction and in leaves its activity was low (Figs. 4, 10). In wheat and barley the enzyme was also more active in the mitochondrial fraction, but only in shoots. On the other hand, in roots the cytoplasmic fraction was more active (Figs. 6, 8). A characteristic feature common for rye, wheat and barley was a drop in the activity on the 5th day of growth in comparison with the 3rd day. Later on, the enzyme activity increased up to the maximum which was reached between the 10th to 21st day, the activity then decreasing again (Figs. 4, 6, 8). The course of enzyme activity in oat plants was similar, with the exception of the initial 10 days, when the activity was very low in all fractions (Fig. 10).

At the stage of milk ripeness the activity of ascorbic acid oxidase in roots and leaves of all four plants was measurable mostly only in the mitochondrial fraction and exhibited lower values in comparison with the initial growth



Figs. 5—10. The variation in cytochrome oxidase (5, 7, 9) and ascorbic acid oxidase (6, 8, 10) activities of wheat (5, 6), barley (7, 8) and oat (9, 10) plants in the course of a 28-day cultivation under artificial conditions. For further caption see Figs. 1 and 2.

phase. It was only in spikes where it was measurable in both fractions and with rye and oat it was somewhat higher than in leaves and roots.

Discussion

Several oxidative enzymes occur in cereal plants. In addition to cytochrome oxidase, peroxidase and ascorbic acid oxidase are also active (WAYGOOD 1950, JAMES 1953, SISAKYAN and VASIL'EVA 1954, HONDA 1955). JAMES (1953) states that cytochrome oxidase occurs only in embryonal barley tissues; after 10 days of growth it seems to be substituted by ascorbic acid oxidase. However, SISAKYAN and FILIPOVICH (1956) and RUBIN and LADYGINA (1956) demonstrated the presence of cytochrome oxidase even in older plant tissues.

It follows from our results that in cereal plants both enzymes investigated are active probably throughout the entire ontogeny. The loss of cytochrome oxidase activity, as observed by JAMES (1953) in barley, was not found. As follows from the comparison of cytochrome oxidase activity in both cell fractions, the enzyme is predominantly concentrated in the mitochondrial fraction. This is in agreement with most literary data on the localization of mechanisms of oxidative phosphorylation, part of which the cytochrome oxidase chain represents, in the inner membrane of mitochondria (HANSEN and SMITH 1964, YASAÏTIS 1973). The enzyme activity demonstrated in the cytoplasmic fraction might be associated *e.g.* with the presence of microsomes which also exhibit a slight cytochrome oxidase activity (ZBARSKIĬ *et al.* 1968), or might be the result of partial diffusion of the enzyme from damaged mitochondria into cytoplasm. Mitochondria are partially damaged during tissue homogenization, and especially due to distilled water used as an isolation medium. PARSONS *et al.* (1966) used the method of hypotonic effect — a shock — for breaking and successive separation of the outer mitochondrial membrane. Intact mitochondria isolated in a medium of suitable tonicity should not respond to exogenous cytochrome *c* (BONNER 1967). As found out by WOJTCZAK and ZALUSKA (1969), the outer mitochondrial membrane is impermeable to cytochrome *c* molecules owing to their large size. From this point of view the method for the isolation of mitochondria in a water medium appears to be suitable for the study of cytochrome oxidase activity in mitochondria.

Our results concerning the localization of ascorbic acid oxidase are not unambiguous. The enzyme was active variably in both cell fractions in dependence on the plant species and organ. In certain cases it appeared to be almost totally concentrated in the mitochondrial fraction, as *e.g.* in shoots of barley and wheat and in rye roots. In other cases, a higher enzyme activity was proved in cytoplasm (wheat and barley roots). The data given in literature mostly deal with the enzyme activity in non-fractionated cell extracts. The demonstration of ascorbic acid oxidase in the mitochondrial fraction of plant tissues is referred to by GRANROTH (1971).

The course of cytochrome oxidase activity during the first month of growth resembles the overall course of respiratory rate as given *e.g.* for barley (JAMES 1953). Beginning from germination the respiratory rate increases up to the maximum, then it diminishes. However, certain differences occur in the time when the maximum was obtained. Maximum enzyme activities were mostly found a little later (10th—14th day) than referred to for respiration, *e.g.* with barley and wheat on the 6th—11th day (JAMES 1953). This may partly be due to the fact that the respiratory and enzyme activities were expressed on different bases (in terms of fresh weight in the case of respiration, per protein unit in that of oxidases).

The course of variation in ascorbic acid oxidase, especially in rye, barley and wheat plants, is analogous to that of the respiratory rate, as well. However, the enzyme activity is lower in comparison with cytochrome oxidase. The drops in ascorbic acid oxidase activity, as determined within the 5th to 10th day of growth with all plants examined, indicate that the enzyme does not participate in the oxidative metabolism at all growth phases. In oat plants this drop was marked also in the case of cytochrome oxidase and maximum activity of both enzymes was obtained as late as on the 21st day

of growth. It is likely that in this case the oxidative metabolism is mediated by further oxidative systems, as *e.g.* by flavin oxidases (ARTSIKHOVSKAYA and RUBIN 1954). This possibility cannot be excluded even in the case of the other plants investigated.

The results obtained suggest that cytochrome oxidase is an active oxidase functioning probably throughout the entire ontogeny of the cereal plants tested. The localization of this enzyme in mitochondria supports the view of its significance in the energetic metabolism of cereal plants.

As far as ascorbic acid oxidase is concerned, some of the results obtained give evidence of the fact that it also plays the role of an oxidative enzyme throughout the whole ontogeny of cereal plants, though to a lesser extent.

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References

ALLEN, P. J.: Physiological aspects of fungus diseases of plants. — Annu. Rev. Plant Physiol. **5** : 225—248, 1954.

ARTSIKHOVSKAYA, E. V., RUBIN, B. A.: [Plant respiration as an adaptable function.] In Russ. — Uspekhi sovrem. Biol. **37** (2) : 136, 1954.

BONNER, W. D.: A general method for the preparation of plant mitochondria. — In: ESTABROOK, R. W., PULLMAN, M. E. (ed.): Methods in Enzymology. Vol. 10. Pp. 126—133. New York 1967.

FARKAS, G. L., KIRÁLY, Z.: Role of phenolic compounds in the physiology of plant deseases and diseases resistance. — Phytopathol. Z. **44** : 105—150, 1962.

GRANROTH, B.: Ascorbic-acid oxidase and phenol-oxidase in quince-d *Cydonia oblonga*-D leaf tissue. — Trans. Mo. Acad. Sci. **5** : 133, 1971.

HANSEN, M., SMITH, A. L.: Studies on the mechanism of oxidative phosphorylation. VII. Preparation of submitochondrial particle (ETP_H) which is capable of fully coupled oxidative phosphorylation. — Biochem. biophys. Acta **81** : 214—222, 1964.

HANUŠOVÁ, M.: Über die Aktivität der Polyphenoloxydasen und Ascorbinsäureoxydase in Apfelblattern, die von *Venturia inaegualis* (CKE) WINT. infiziert waren. — Phytopathol. Z. **65** : 189—200, 1969.

HONDA, S. I.: Ascorbic acid oxidase in barley roots. — Plant Physiol. **30** : 174—181, 1955.

JAMES, W. O.: Plant Respiration. — Clarendon Press, Oxford 1953.

LEHNINGER, A. L.: The Mitochondrion. Molecular Basis of Structure and Function. — New York—Amsterdam 1965.

LOWRY, P. H., ROSENBROUGH, J. N., FARR, L. A., RANDALL, R. J.: Protein measurement with the Folin phenol reagent. — J. biol. Chem. **193** : 265—275, 1951.

MILLIKAN, D. F., SANIEWSKI, M.: Phenolase and ascorbic acid oxidase activities in apple leaves after grafting with healthy and virus infected Golden Delicious buds. — Phytopathology **62** : 778, 1972.

PAECH, K., TRACEY, M. V.: Moderne Methoden der Pflanzenanalyse. — Vol. IV. Springer Verlag, Berlin—Göttingen—Heidelberg 1955.

PARSONS, D. F., WILLIAMS, G. R., CHANCE, B.: Characteristics of isolated purified and preparations of the outer and inner membranes of mitochondria. — Ann. N. Y. Acad. Sci. **137** : 643 to 666, 1966.

PETINOV, N. S., MALYSHEVA, K. M.: [Effect of drought on the effectiveness of maize leaf respiration.] In Russ. — Fiziol. Rast. **7** : 553—557, 1960.

PLHÁK, F.: Respirační metabolismus rostlin cukrovky při společném růstu s pýrem. [Respiratory metabolism of sugar beet plants grown along with couch grass.] — Folia Fac. Sci. nat. Univ. purkynianae brunensis **6** : 1—92, 1965.

RUBIN, B. A., ARTSIKHOVSKAYA, E. V.: Biokhimiya i Fiziologiya Immuniteta Rasteniĭ. [Biochemistry and Physiology of Plant Immunity.] In Russ. — Moskva 1960.

RUBIN, B. A., LADYGINA, M. E.: [Characterization of plant cytochrome oxidase.] In Russ. — Biokhimiya **21** : 347—355, 1956.

SISAKYAN, N. M., FILIPOVICH, I. I.: [Localization of cytochrome oxidase in a plant cell.] In Russ. — Biokhimiya **21** : 163—173, 1956.
SISAKYAN, N. M., VASIL'EVA, N. A.: [Oxido-reduction processes in durum and common wheats.] In Russ. — Biokhimiya **19** : 730—734, 1954.
WAYGOOD, E. R.: Physiological and biological studies in plant metabolism. — Can. J. Res. **28** C : 7—62, 1950.
WOJTCZAK, L., ZALUSKA, H.: On the impermeability of the outer mitochondrial membrane to cytochrome *c*. I. Studies on whole mitochondria. — Biochem. biophys. Acta **193** : 64—72, 1969.
YASAÏTIS, A. A.: [Inner membrane of mitochondria. Morphological, structural and functional aspects.] In Russ. — In: SERGEEV, P. V. (ed.): Biologicheskie Membrany. Moskva 1973.
ZBARSKIĬ, I. B., POKROVSKII, A. A., PEREVOSHCHIKOVA, K. A., GAPPAROV, M. M., DELEKTORSKAYA, L. N., LASHNEVA, N. V.: [Oxidative enzymes in nuclear membranes of rat liver.] In Russ. — Dokl. Akad. Nauk SSSR **181** : 993—996, 1968.

N. RŮŽIČKOVÁ-SKŘIPSKÁ (Brno): **Aktivita cytochromoxidázy a askorbázy v rostlinách obilovin.** — Biol. Plant. **18** : 36—43, 1976.

V práci jsme studovali změny aktivity cytochromoxidázy a askorbázy v rostlinách žita, pšenice, ječmene a ovsa. Aktivita obou enzymů byla měřena v průběhu počátečních 28 dnů růstu a ve fázi mléčné zralosti v cytoplazmatické a mitochondriální buněčné frakci kořenů, listů a klasů. Oba enzymy byly aktivní při všech měřeních. Cytochromoxidáza vykazovala většinou vyšší aktivitu než askorbáza. Cytochromoxidáza byla v listech, kořenech i klasech všech čtyř pokusných rostlin podstatně aktivnější v mitochondriální frakci ve srovnání s cytoplasmatickou frakcí. Aktivita askorbázy v obou frakcích se naopak měnila podle druhu rostliny, druhu orgánu i podle fáze růstu.

Změny aktivity obou enzymů během růstu rostlin vykazovaly vcelku podobný průběh změnám intenzity respirace. V prvých 14 až 21 dnech růstu se aktivita enzymů zvyšovala k maximu, poté zprvu rychleji, později pozvolna klesala. Průběh změn aktivity enzymů byl s určitými výjimkami podobný u všech čtyř sledovaných rostlin.

BOOK REVIEW

JACOBI, K.: **Hausbuch der Zimmerpflanzen. Sonderausgabe.** — BLV Verlagsgesellschaft, München—Bern—Wien 1975. 190 S. 14,— DM.

Hiermit erscheint schon die 5. Auflage des beliebten Hausbuches aller Zimmerpflanzenfreunde. Das Buch ist in vier Abschnitte gegliedert. Der erste bringt an Hand von illustrativen Zeichnungen die Zusammenstellung von Zimmerpflanzen für Süd- und Nordfenster, Kakteen- oder Sukkulentengruppen, buntblättrige oder blühende Blumenfenster, anspruchslose Zimmerpflanzen u.a.m. Der zweite, ausführlichste Abschnitt „Zimmerpflanzen im Porträt" ist ein alphabetisches Verzeichnis von mehr als 120 Zimmerpflanzen je mit Zeichnung und Charakteristik der Pflanze. Es folgen einige spezielle Kapitel über Zwiebelpflanzen, Kübel- und Balkonpflanzen, Wasserpflanzen usw. Der dritte Abschnitt ist der richtigen Pflege der Zimmerpflanzen und der Krankheitsbekämpfung gewidmet. Der vierte Abschnitt behandelt kurz Zimmerpflanzen mit besonderen Lebensansprüchen, die Vermehrung der Zimmerpflanzen, die Hydrokultur u.ä. Das Buch schliesst mit einem Pflanzen- und Sachverzeichnis. Ausser vielen schwarz-weissen und schwarz-weiss-grünen Zeichnungen enthält das Buch ebenfalls gute Schwarz-Weiss- und Farb-Fotos (Das Foto der Azalee ist nicht auf S. 69 — siehe Textanweisung auf S. 107 — sondern auf S. 123 zu finden).

Als Ganzes ist das Büchlein sehr gelungen und wird meines Erachtens vielen Blumenfreunden gute Dienste leisten.

INGRID TICHÁ (*Praha*)