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The relationship between respiration rate and peroxidase activities in maize root mitochondria

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Abstract

In the present study we provide the evidence of different respiration rates and peroxidase activities in maize (*Zea mays* L.) mitochondria isolated from germinated seeds and roots of 2-week-old seedlings. The negative relationships between mitochondrial respiration rate measured with NADH as substrate and activities of peroxidases that oxidized NADH in both oxidative and peroxidative cycles were found. The possible role of peroxidase in the regulation of reactive oxygen species metabolism in expense of NADH oxidation was hypothesized.

Additional key words: alternative oxidase, reactive oxygen species, *Zea mays*.

Introduction

Plant mitochondria generate reactive oxygen species (ROS). The rate of the mitochondrial free radical production increases as a result of the diminished electron transport through cytochrome part of respiratory chain. The main sites of this production are the respiratory complexes I and III (Turens *et al*. 1985, Chen *et al*. 2003). Because ROS can cause damage to proteins, lipids and DNA, their production and removal must be strictly controlled. In the normally grown plants, ROS are readily removed by mitochondrial antioxidant systems. In addition to some enzymatic detoxification systems, such as peroxidase (POD), superoxide dismutase (SOD), catalase, *etc*., the alternative oxidase (AOX) represents the mechanism for lowering the ROS production in plant mitochondria (Maxwell *et al*. 1999).

The plant respiratory chain has two pathways of the electron transport that branches at the ubiquinone: the cytochrome pathway coupled to the ATP synthesis (KCN-sensitive), and the alternative pathway (which consists of the enzyme AOX) non-coupled to the ATP production and responsible for cyanide resistant respiration. In condition of the limited cytochrome chain activity, AOX provides an alternative path for the transport of electrons from ubiquinol to molecular oxygen to form water, without the release of superoxide or hydrogen peroxide. Therefore, one of its functions may be to prevent the overreduction of the respiratory ubiquinone pool at the inner mitochondrial membrane and also to decrease the concentration of $O_2^{\bullet -}$, providing protection against the ROS formation (Purvis and Shwefelt 1993, Wagner and Krab 1995, Maxwell *et al*. 1999). The alternative pathway activity is affected by the developmental stage, tissue type, physiological status and environmental conditions (Laties 1982, Shimoji and Yamasaki 2005). Both the AOX protein content and reduction of the ubiquinone pool are important regulators of the AOX activity. Furthermore, the AOX activity may be regulated by the redox state of a disulphide bond between the two subunits of the homodimeric enzyme (Umbach and Siedow 1993), which can be regulated in intact mitochondria by the redox poise of the NADP(H) pool in the mitochondrial matrix (Vanlenberghe *et al*. 1995). On the other hand, more active reduced form of AOX can be stimulated by pyruvate and some other oxo-keto acids, which act as the allosteric activators (Millar *et al*. 1993, 1996).

Mitochondrial PODs from maize root are similar to the class III peroxidases (EC 1.11.1.7) and can be considered as bifunctional enzymes with characteristics of both, peroxidase and oxidase activities. In the classical

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Abbreviations: AOX - alternative oxidase; CoQ - coenzyme Q; DTT - dithiotreitol; ETC - electron transport chain; HDM - higher density mitochondria; LDM - lower density mitochondria; M - mitochondria; POD - peroxidase; PVP - polyvinylpyrrolidone; ROS - reactive oxygen species; SOD - superoxide dismutase; UQ_r - reduced ubiquinone.

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peroxidase cycle, they use H_2O_2 as an oxidant to oxidize a variety of substrates (Prasad *et al*. 1995), including numerous phenolic substances. Because H_2O_2 is removed during the reactions of phenolic substrates oxidation, PODs are considered as antioxidant enzymes, which protect cells from the destructive influence of H_2O_2 and derived oxygen species. Additionally, in the presence of a suitable reductant such as NADH, PODs may constitute H_2O_2/phenolic/NADH system for H_2O_2 scavenging (Hadži-Tašković Šukalović *et al*. 2003). In this system, phenoxyl radicals formed by the POD catalyzed oxidation of phenolics, cooxidized NADH acting as a secondary and ultimate reductant. In the oxidative cycle, in the presence of NAD(P)H, mitochondrial POD(s) can also generate H_2O_2 mediating the reduction of O_2 to $O_2^{\cdot-}$ (Hadži-Tašković Šukalović and Vuletić 2003). Although the capability of mitochondrial POD to oxidize NADH in both enzyme cycles was recently demonstrated *in vitro*, and enzyme characteristics distinct from the known electron transport enzymes were documented, their physiological role is still unknown.

Having in mind that alteration of the level of NADH in mitochondria could be achieved by POD reactions, in addition to the membrane NADH dehydrogenases, in this paper we have presented the possible relationship between POD activity and mitochondrial respiration.

Materials and methods

Plants and growth conditions: Maize (*Zea mays* L.) inbred line VA35 seeds were germinated for 3 d and then they were transferred into plastic pots containing Knopp solution, modified in the nitrogen content. The first 7 d plants were grown on ¼ strength nutrient solution and during the following 4 d on full strength solution. Nitrogen was supplied as KNO_3, $Ca(NO_3)_2$ and $(NH_4)_2SO_4$. The concentration of NO_3^- and NH_4^+ in full strength solution was 10.9 and 7.2 mM, respectively. The initial pH of the solution was adjusted to 5.6. Plants were kept in a growth chamber under a 12-h photoperiod at 22/18 °C, with the irradiance of 40 W m^{-2} and relative humidity of 70 %.

In addition, in some experiments, maize seedlings were grown for 4 d in the dark on moistened filter paper.

Isolation and purification of mitochondria: Mitochondria were prepared by a modified procedure of Schwitzguebel and Siegenthaler (1984). Roots were cut in five volumes of medium containing 0.4 M mannitol, 50 mM TES buffer (pH 7.5), 40 mM cysteine, 1 mM EDTA, 1 mM $MgCl_2$, 0.1 % (m/v) BSA and 1 % (m/v) soluble polyvinylpyrrolidone (PVP), and ground with a mortar and pestle. After filtration through 4 layers of muslin, centrifugation and washing steps were done as described by Hadži-Tašković Šukalović and Vuletić (2001). Washed mitochondria were purified on a discontinuous Percoll gradient made of five layers prepared with 5 cm^3 50 %, 10 cm^3 45 %, 5 cm^3 27 %, 5 cm^3 20 % and 5 cm^3 13.5 % Percoll, with PVP added (Hadži-Tašković Šukalović and Vuletić 2001).

The band of mitochondria at the 27/45 % interface and pellet from the bottom of the centrifugal tube were collected. Mitochondria were used for the experiments immediately after washing and centrifugation. Possible contamination with microbodies and plastids were determined by analyzing activities of marker enzymes, catalase (EC 1.11.1.6) according to Aebi (1974) and phosphogluconate dehydrogenase (EC 1.1.1.44) according to Journet and Douce (1985), as previously described (Hadži-Tašković Šukalović and Vuletić 1998).

In order to determine the intactness of mitochondria, the activity of succinate: cytochrome *c* oxidoreductase (EC 1.9.3.1), in the absence and in the presence of 0.025 % (v/v) Triton X-100, was measured (Tolbert 1974). Fresh isolates with integrity of outer membrane over 90 % or frozen mitochondria were used for respiratory experiments and POD activity determination, respectively.

Oxygen consumption measurements: Oxygen consumption was measured with a Clark-type O_2 electrode (*Hansatech Ltd*., King's Lynn, England) at 25 °C, in a 1 cm^3 reaction medium containing 0.05 to 0.1 mg mitochondrial protein, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 0.4 M mannitol and 1 mg cm^{-3} bovine serum albumin in 10 mM (3-[N-morpholino] propane-sulfonic acid) buffer, pH 7.2. State 3 respiration was triggered by the addition of 0.25 mM ADP.

Respiration of isolated mitochondria was measured with various substrates: 10 mM succinate, 1 mM NADH, or both, succinate plus NADH (Holtzapffel *et al*. 1998), in order to saturate the electron transport chain. KCN (1 mM) was used as inhibitor of the cytochrome oxidase and n-PG (0.25 mM) as inhibitor of the AOX. Wherever succinate was used as a substrate 0.1 mM ATP was used as an activator. The AOX activity was determined as a cyanide resistant, n-PG sensitive activity, and was calculated as the difference between the slopes just before and after n-PG addition, in the presence of KCN (Liden and Åkerlund 1993).

Determination of peroxidase (EC 1.11.1.7) activities: The oxidative activity of POD was determined in 50 mM phosphate buffer at pH 5.5 by monitoring the decrease of NADH absorbance at 340 nm in the assay mixture containing 0.3 mM NADH, 0.2 mM *p*-coumaric acid, 0.25 mM $MnCl_2$, 0,025 % Triton X-100 and 2.5 - 5 μg of a mitochondrial protein in a volume of 1 cm^3.

The standard guaiacol test (Chance and Maehly 1955) for determination of peroxidative activity of POD, by measuring absorbance at 436 nm in the presence of H_2O_2, 0.025 % Triton X-100 and 10 μg of the mitochondrial protein in 1 cm^3 of 50 mM phosphate buffer at pH 6.5, was used.

Activity of POD was determined with various phenolic substrates, by measuring the initial rate of the decrease of absorbance at a corresponding maximum in 1 cm^3 assay mixture containing 0.1 mM phenolic, 2.5 mM H_2O_2 and 5.0 μg of mitochondrial protein, in 50 mM phosphate buffer pH 6.0, (Hadži-Tašković Šukalović and Vuletić 2003). The rate of phenolic oxidation without H_2O_2 was measured as a control.

NADH oxidation by the POD/phenolic system was measured at 340 nm in 1 cm^3 assay mixture containing 50 mM phosphate buffer pH 6.0, 0.025 % Triton X-100, equimolar concentrations of NADH and phenolic (0.2 mM), 2.5 mM H_2O_2 and 10-20 μg of mitochondrial protein.

The mitochondrial protein content was measured in the presence of 0.005% Triton X-100 by the method of Lowry *et al*. (1951) with bovine serum albumin as a standard.

Results

Mitochondrial populations: Mitochondria isolated from maize roots were purified on the discontinuous Percoll density gradient with PVP added in order to achieve better separation (Hadži-Tašković Šukalović and Vuletić 1998). Isolates from 2-week-old maize roots were separated into several density bands. Two bands with the integrity of outer membrane >90 % were used for further experiments: lower density (LDM), appearing in the gradient at 27/45 % Percoll interface, and higher density mitochondria (HDM) at the bottom of the gradient. Intact mitochondria from seedling roots (M) were homogeneous and appeared at the 27/45 % interface as a less dense fraction. An additional, mitochondrial fraction appearing in both samples at the interface 20/27 % was discarded because it exhibited a low outer membrane integrity (data not presented). Low specific activities of catalase and phosphogluconate dehydrogenase (data not presented), excluded the effect of contaminations of our isolates with peroxisomes and plastids, respectively.

Respiration rate: Mitochondria from seedling roots oxidized substrates: succinate, NADH or both (succinate + NADH) at higher rates than mitochondria from 2-week-old roots. The respiration rate was lower in LDM fraction than in HDM fraction obtained from the same crude isolate from 2-week old roots. In all mitochondrial isolates the rate of the O_2 uptake (State 4) with NADH was higher than with succinate (Table 1).

The AOX activity was assayed as the oxygen uptake with NADH, succinate or both, in the presence of KCN. The activity was the highest in mitochondria isolated from seedling roots, with either substrate used for respiration (Fig. 1). To examine the activation effect of DTT and pyruvate, during the respiratory measurement with succinate plus NADH, pyruvate was added following DTT. DTT alone was ineffective in any mitochondrial isolate (data not presented). Stimulation with pyruvate (about 73 %) was achieved only in mitochondria isolated from seedling roots (Fig. 1). Although total respiration was more intensive with NADH than succinate (Table 1), lower activities of alternative oxidase during NADH oxidation were observed (Fig. 1). In all of the experiments the HDM fraction showed a higher AOX activity in comparison to the LDM fraction (Fig. 1).

Table 1. Respiration rates [μmol(O_2) g^{-1}(protein) min^{-1}] of mitochondria isolated from maize seedling roots (M) and 2-week-old roots (LDM and HDM), measured with various substrates. State 3 respiration was triggered by the addition of 0.25 mM ADP. Means ± SE are shown with a number of isolations indicated within parentheses.

Substrate	State	M	LDM	HDM
Succinate	4	153±27 (2)	89±18 (3)	141±29 (3)
NADH	4	210± 5 (2)	111±10 (3)	170±47 (3)
Succinate	3	461±59 (2)	236±17 (3)	387±42 (2)
+NADH	4	213±14 (2)	121±18 (3)	207±10 (2)

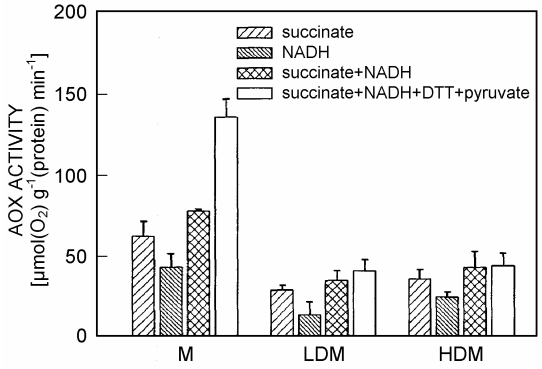

Fig. 1. AOX activity assayed as oxygen uptake with various substrates in the presence of KCN, added following the substrates. In the experiment with DTT (2.5 mM) and pyruvate (10 mM), they were added after KCN. The *error bars* represent SE of at least two isolates.

Peroxidase activities: Mitochondria isolated from 2-week-old roots exhibited higher oxidative, as well as, peroxidative activities, either with guiacol or with various phenolic substrates, than mitochondria isolated from seedling roots (Table 2). These activities were higher in LDM than in HDM fraction of 2-week-old roots.

Experiments with natural phenolic compounds were performed without Triton X-100 in order to avoid its high absorbance in the UV range in which spectra changes were recorded. Because of high latency of POD activity (Hadži-Tašković Šukalović and Vuletić 2003), the values for phenolic peroxidases in the presence of Triton X-100

Table 2. The specific activities of the oxidative [mmol(NADH oxidized) g^{-1}(protein) min^{-1}] and peroxidative cycle [mmol(tetraguaiacol produced) g^{-1}(protein) min^{-1}] or [mmol(phenolic oxidized) g^{-1}(protein) min^{-1}] in mitochondria isolated from seedling roots and 2-week-old maize roots. Means ± SE of at least two isolations. nd - not detectable.

	Substrate	M	LDM	HDM
Oxidative activity	NADH	2.43±0.2	17.60±0.10	7.29±0.03
Peroxidative activity	guaiacol	0.51±0.0	4.35±0.17	1.67±0.08
	coniferyl alcohol	nd	1.90±0.02	0.93±0.53
	caffeic acid	nd	0.83±0.02	nd
	ferulic acid	nd	0.63±0.01	nd
	p-coumaric acid	nd	0.35±0.01	nd

did not represent maximum enzyme activities. The results showed that mitochondrial POD oxidized coniferyl alcohol more readily than phenolic acids, and demonstrated descending order of substrate preference: coniferyl alcohol > caffeic acid > ferulic acid > *p*-coumaric acid. All the assays were performed at optimal pH 6.0, determined for each phenolic substrate (data not presented).

Respiration rates of mitochondrial isolations (total respiration rate or AOX) exhibited a negative correlation to their POD activities. These relationships were similar in the case of both, peroxidative (Fig. 2*A*) and oxidative cycle (Fig. 2*B*) activities of POD, being more pronounced in the case of the total respiration rate than for the AOX activity.

To compare the efficiency of the different phenolics to constitute *in vitro* H_2O_2/phenolic/NADH system, we used phenolics and NADH in equimolar concentrations. It was shown that mitochondrial preparations from

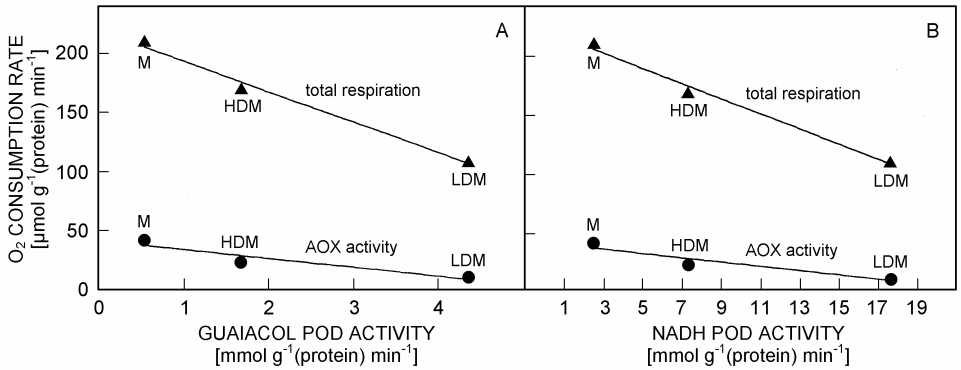

Fig. 2. Relationship between peroxidase activities in the oxidative (A) and peroxidative (B) cycle, and mitochondrial respiration rates (total and alternative pathway) in state 4, with NADH as a substrate in mitochondrial preparations isolated from maize seedling roots (M) and 2-week-old roots (LDM and HDM).

Table 3. Rates of NADH oxidation [mmol (NADH) g^{-1}(protein) min^{-1}] by mitochondria isolated from 2-week old maize roots in the presence of H_2O_2 and various phenolic compounds. Means ± SE of two isolations.

Phenolic compound	LDM	HDM
Coniferyl alcohol	0.91±0.17	0.23±0.05
Caffeic acid	3.79±0.20	0.49±0.00
Ferulic acid	0.85±0.02	0.19±0.02
p-Coumaric acid	1.65±0.03	0.24±0.03

2-week-old roots used NADH as a secondary oxidant with all of the tested phenolic (Table 3), while mitochondria isolated from seedling roots did not show detectable amounts of NADH oxidation in such experiments. The rate of NADH oxidation was higher with the LDM than with the HDM fraction, being in agreement with generally higher activities of POD (Table 2). However, the preferences for phenolics in NADH co-oxidation reaction, with descending order: caffeic acid > *p*-coumaric acid > coniferyl alcohol > ferulic acid, was different in comparison to preference for phenolics as substrates in peroxidative POD reaction.

Discussion

Our results demonstrated that maize root mitochondria isolated from seedlings and 2-week-old roots were different in respect to the density. The appearance of higher density fraction in older tissue, which has been already shown (Hadži-Tašković Šukalović and Vuletić 2001), could be the characteristic of root development. Our previous results demonstrated that the activities of several key enzymes of the tricarboxylic acid (TCA) cycle, which can be considered as non-ETC mitochondrial markers, were at similar level in LDM and HDM (Hadži-Tašković Šukalović and Vuletić 2001). However, mitochondrial fraction from maize seedlings,

as well as two fractions from 2-week old roots, obtained in present work, exhibited different respiratory and POD activities.

From the results presented, it is obvious that the total mitochondrial respiration rate is higher in seedlings than in 2-week-old roots. The decrease of mitochondrial respiration rate during plant growth was also shown for different plant tissues (McDonnel and Farrar 1993, Millar *et al*. 1998). These results are in accordance with previous results showing that rapidly growing tissues actively respire in order to provide a high contents of ATP, reductants and carbon skeleton for cell division and expansion (Lambers *et al*. 1983). We also showed that, with all used respiratory substrates, the activity of AOX declined with maize root age, following the total respiration pattern, although its participation in the total respiration remained almost constant. The opposite results were obtained for other studied plant objects, such as soybean seedling roots (Millar *et al*. 1998) and cotyledons (McCabe *et al*. 1998), or tobacco suspension cells (Vanlerberghe and McIntosh 1992).

On the other hand, using these mitochondrial isolates with different respiration rates to study their oxidative and peroxidative activities of PODs, we demonstrated a negative correlation of both POD activities to the total respiration rate and to the AOX activity, with NADH as the substrate. According to our results obtained *in vitro*, showing almost a constant ratio of the oxidative/ peroxidative activity, it is possible that both POD cycles operate simultaneously. Since both POD cycles, oxidative and peroxidative (as H_2O_2/phenol/NADH system), oxidize NADH, their role in maintaining the NAD^+/ NADH ratio and keeping the matrix NAD pool relatively oxidized, as well as decreasing O_2 concentration in mitochondria, could be hypothesized. It could be supposed that a regulation of the reduction level of ETC by means of such non-coupled NADH oxidation (independent of ETC) leads to the regulation of the UQ redox state and consequently, of the AOX activity. Also, decrease of AOX activity can be achieved by direct oxidation of the UQ_{red} with $O_2^{\bullet-}$ or H_2O_2 produced in the oxidative cycle of POD.

Mitochondrial PODs were shown to play a role in the production, as well as, in scavenging the H_2O_2 (Hadži-Tašković Šukalović and Vuletić 2003). The increased activity of POD in mitochondrial isolates with lower respiration rates, as shown in our experiments, suggested that non-coupled NADH oxidation by maize root mitochondria POD could be involved as an additional mechanism in ROS metabolism in that case. Previously published results argue in favour of the role of AOX in the regulation of the ROS level, showing the inverse relationship between the AOX activity and the H_2O_2 generation by mitochondria (Braidot *et al*. 1999) and stimulation of ROS production by inhibition of AOX or cytochrome pathway (Popov 2003). However, our results, which demonstrated the decreased AOX activity in mitochondrial fractions with decreased total respiration rates and stimulation of KCN-insensitive respiration with exogenous pyruvate only in young mitochondria, indicated that AOX might be dominant in the regulation of the ROS level only in the seedling stage with a high respiration rate. On the other hand, high POD activities in older tissues with a low AOX activity indicated that the restricted activity of AOX could be compensated by increased POD activity. Since POD enzyme is located within mitochondria (Prasad *et al*. 1995, Hadži-Tašković Šukalović and Vuletić 2003), its reaction products in the oxidative reaction, $O_2^{\bullet-}$ and H_2O_2, are probably directed towards the matrix antioxidant defense systems. A low catalase activity (Hadži-Tašković Šukalović and Vuletić 1998, 2003), having high K_m for H_2O_2 (Baker *et al*. 2000), and a high peroxidative activity in 2-week old root mitochondria, suggest that POD activity could play a dominant role in H_2O_2 scavenging by using H_2O_2 as a substrate for oxidation of various phenolic substrates, or more probably, for cooxidation of NADH, through the H_2O_2/phenol/NADH system. Recent findings of Mittova *et al.* (2004) confirmed that peroxidases are the main H_2O_2 scavengers in root mitochondria.

In conclusion, based on the comparison of POD activities of different mitochondrial fractions with their respiration rates, it was suggested that POD activities could play a role in the developmental regulation of the ROS metabolism in maize root mitochondria. It seems possible that, in the condition of the decreased respiration, the increased ROS production could be achieved through the oxidative cycle of POD. Also, H_2O_2 scavenging, beside well-known mitochondrial antioxidative defense mechanisms, may possibly be achieved through POD reactions.

References

Aebi, H.: Catalase. - In: Bergmeyer, H.U. (ed): Methods in Enzymatic Analysis. Pp. 764 -767 Verlag Chemie, Weinheim 1974.

Baker, C.J., Deahl, K., Domek, J., Orlandi, E.W.: Scavenging of H_2O_2 and production of oxygen by horseradish peroxidase. - Arch. Biochem. Biophys. **382**: 232-237, 2000.

Braidot, E., Petrussa, E., Vianello, A., Macri, F.: Hydrogen peroxide generation by higher plant mitochondria oxidizing complex I or complex II substrates. - FEBS Lett. **451**: 347-350, 1999.

Chance, B., Maehly, A.C.: Assay of catalase and peroxidases. - Methods Enzymol. **2**: 764-775, 1955.

Chen, Q., Vazquez, E.J., Moghaddas, S., Hoppel, C.L., Lesnefsky, E.J.: Production of reactive oxygen species by mitochondria. Central role of complex III. - J. biol. Chem. **278**: 36027-36031, 2003.

Hadži-Tašković Šukalović, V., Vuletić, M.: Properties of maize root mitochondria from plants grown on different nitrogen sources. - J. Plant Physiol. **153**: 67-73, 1998.

Hadži-Tašković Šukalović, V., Vuletić, M.: Heterogeneity of

maize root mitochondria from plants grown in the presence of ammonium. - Biol. Plant. **44**: 101-104, 2001.

Hadži-Tašković Šukalović, V., Vuletić, M.: The characterization of peroxidases in mitochondria of maize roots. - Plant Sci. **164**: 999-1007, 2003.

Hadži-Tašković Šukalović, V., Vuletić, M., Vučinić, Ž.: Plasma membrane-bound phenolic peroxidase of maize roots: in vitro regulation of activity with NADH and ascorbate. - Plant Sci. **165**: 1429-1435, 2003.

Holtzapffel, R.C., Finnegan, P.M., Millar, A.H., Dutilh, B., Badger, M.R., Day, D.A.: Purification and properties of mitochondria from tomato. - In: Møler, I.M., Gardeström, P., Glimelius, K., Glaser, E. (ed.): Plant Mitochondria: From Gene to Function. Pp. 469-473. Backhuys Publishers, Leiden 1998.

Journet, E.P., Douce, R.: Enzymatic capacities of purified cauliflowers bud plastids for lipid synthesis and carbohydrate metabolism. - Plant Physiol. **79**: 458-463, 1985.

Lambers, H., Szaniawski, R.K., De Visser, R.: Respiration for growth, maintenance and ion uptake: an evaluation of concepts, methods, values and their significance. - Physiol. Plant. **58**: 556-563, 1983.

Laties, G.G.: The cyanide-resistant, alternative path in higher plant respiration. - Annu. Rev. Plant Physiol. **33**: 519-555, 1982.

Liden, A.C., Åkerlund, H.-E.: Induction and activation of the alternative oxidase of potato tuber mitochondria. - Physiol. Plant. **87**: 134-141, 1993.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Maxwell, D.P., Wang, Y., McIntoch, L.: The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. - Proc. nat. Acad. Sci. USA **96**: 8271-8276, 1999.

McCabe, T.C., Finnegan, P.M., Millar, A.H., Day, D.A., Whelan, J.: Differential expression of alternative oxidase genes in soybean cotyledons during postgerminative development. - Plant Physiol. **118**: 675-682, 1998.

McDonnel, E., Farrar, J.F.: Respiratory characteristics of isolated barley mitochondria and intact barley roots. - J. exp. Bot. **44**: 1485-1490, 1993.

Millar, A.H., Wiskich, J.T., Whelan, J., Day, D.A.: Organic acid activation of the alternative oxidase of plant mitochondria. - FEBS Lett. **329**: 259-262, 1993.

Millar, A.H., Hoefnagel, M.H.N., Day, D.A., Wiskich, J.T.: Specificity of the organic acid activation of alternative oxidase in plant mitochondria. - Plant Physiol. **111**: 613-618, 1996.

Millar, A.H., Atkin, O.K., Menz, R.I., Henry, B., Farquhar, G., Day, D.A.: Analysis of respiratory chain regulation in roots of soybean seedlings. - Plant Physiol. **117**: 1083-1093, 1998.

Mittova, V., Guy, M., Tai, M., Volokita, M.: Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. - J. exp. Bot. **55**: 1105-1113, 2004.

Popov, V.N.: Possible role of free oxidation processes in the regulation of reactive oxygen species production in plant mitochondria. - Biochem. Soc. Trans. **31**: 1316-1317, 2003.

Prasad, T.K., Anderson, M.D., Stewart, C.R.: Localization and characterization of peroxidases in the mitochondria of chilling-acclimated maize seedlings. - Plant Physiol. **108**: 1597-1605, 1995.

Purvis, A.C., Shewfelt, R.L.: Does the alternative pathway ameliorate chilling injury in sensitive plant tissues. - Physiol. Plant. **88**: 712-718, 1993.

Schwitzguebel, J.P., Siegenthaler, P.A.: Purification of peroxisomes and mitochondria from spinach leaf by Percoll gradient. - Plant Physiol. **75**: 670-674, 1984.

Shimoji, H., Yamasaki, H.: Inhibitory effects of flavonoids on alternative respiration of plant mitochondria. - Biol. Plant. **49**: 117-119, 2005.

Tolbert, N.E.: Isolation of subcellular organelles of metabolism on isopicnic sucrose gradients. - Methods Enzymol. **31**: 734-746, 1974.

Turens, J.F., Alexandre, A., Lehninger, A.L.: Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. - Arch. Biochem. Biophys. **237**: 408-414, 1985.

Umbach, A.L., Siedow, J.N.: Covalent and non-covalent dimmers of the cyanide- resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. - Plant Physiol. **103**: 845-854, 1993.

Vanlenberghe, G.C., McIntoch, L.: Coordinate regulation of cytochrome and alternative pathway respiration in tobacco. - Plant Physiol. **100**: 1846-1851, 1992.

Vanlenberghe, G.C., Day, D.A., Wiskich, J.T., Vanlenberghe, A.E., McIntoch, L.: Alternative oxidase activity in tobacco leaf mitochondria. - Plant Physiol. **109**: 353-361, 1995.

Wagner, A.M., Krab, K.: The alternative respiration pathway in plants: role and regulation. - Physiol. Plant. **95**: 318-325, 1995.