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THE OXIDATIVE PROPERTIES OF MITOCHONDRIA AND BACTEROIDS FROM ROOT NODULES OF SOYBEAN TREATED WITH ORGANIC ACIDS

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Summary. Nodule mitochondria are highly sensitive to the respiratory inhibitor antimycine A. The antimycine-resistant oxygen uptake is 5–10% of the rate of control mitochondrial respiration. The high sensitivity to this inhibitor means that non-phosphorilating pathways are absent from the nodules and the energetic effectiveness of mitochondria is very high. The latter is proved by the good respiratory control, observed in mitochondria from soybean plant nodules. Mitochondria, isolated from nodules of plants that are treated with succinate and α-ketoglutarate during inoculation showed highest respiratory control.

A significant differences in the rate of oxygen uptake by mitochondria isolated from soybean plant nodules were observed in all treatments investigated. The rates of oxygen consumption by mitochondria isolated from nodules of citrate treated plants were close to these of the control plants. However, treatment with succinate, malate and α-ketoglutarate resulted in a significant increase of mitochondrial oxygen uptake.

Our results support the relation between plant photosynthesis and bacteroid respiration. Photosynthetic intensity and the oxygen uptake of bacteroids were the lowest in control soybean plants and the ones treated with citrate during inoculation. In the cases when the intensity of photosynthesis was high (treatments with succinate, malate and α-ketoglutarate during inoculation), the rate of oxygen uptake of the bacteroids was the highest.

Results obtained indicate that the oxidative capacity of mitochondria and bacteroids from root nodules of soybean treated with organic acids succinate and

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α-ketoglutarate was stimulated and this may lead to more effective nodule nitrogen fixation.

Key words: bacteroids, mitochondria, nodules, organic acids, oxygen uptake, respiratory control

Abbreviations: AO – alternative oxidase, Cyt – cytochrome, PBM – peribacteroid membrane

Introduction

Cytosolic ATP is a substrate for both protein kinase and ATPase on peribacteroid membrane (PBM) and is therefore likely to play a key role in the regulation of metabolite exchange across this membrane. Adding ATP to isolated symbiosomes can stimulate malate uptake across the PBM (Ou Yang at al., 1990, 1991). ATP concentration is also important for ammonium efflux, because large quantities are required in plant cytoplasm for ammonium assimilation. Most of plant cell's ATP synthesis occurs in the mitochondria. It is also possible that mitochondria play a role in the production of α-ketoglutarate as amine acceptor in NH_3 assimilation reaction sequence.

Organic acids, especially succinate and malate, are the major sources of carbon supplied *in vivo* to support nodule nitrogenase activity of legumes (O'Brian and Maier, 1989). The mitochondria of nodule cells are also likely to participate in the production of these organic acids. Therefore, mitochondria are very important for the nitrogen-fixing activity of soybean root nodules.

Results of our earlier investigations (Ignatov and Vassileva, 2000) showed that the organic acids malate, succinate, α-ketoglutarate and citrate at 200 μM concentration significantly altered the symbiotic characteristics (nodulation, nodule and plant biomass accumulation and nitrogenase activity) of *Bradyrhizobium japonicum* strain 273 – *Glycine max* nitrogen-fixing system. An increase in the photosynthetic intensity and transpiration in this nitrogen-fixing system was established (Ignatov and Vassileva, 1999). In this paper the oxidative properties of mitochondria and bacteroids from root nodules of soybean treated with organic acids succinate, malate, α-ketoglutarate and citrate are studied.

Materials and Methods

Plant material and bacterial strain

Soybean (*Glycine max* L. cv. Hodgson) seeds were surface sterilized with 70% ethanol (v/v) for 60 min and germinated for 5 days at 25 °C. Sterile seedlings were transferred

to plastic growth pots (1.2 l) with 2 plants per pot and cultivated on Hellriegel's solution (1898) and micronutrients (Hoagland and Arnon, 1950) with 21 mg.l^{-1} nitrogen in a naturally illuminated greenhouse at sterile conditions as described by Ignatov et al. (2000). Soybean seedlings were inoculated with *Bradyrhizobium japonicum* strain 273, using inoculant dose of 10^8 viable cells. The strain was cultivated and maintained on yeast-mannitol (YM) medium (Vincent, 1970) at 28 °C. Organic acids were added to the growth medium at final concentration of 200 µM. Nodules were harvested 8 weeks after planting and were used immediately.

Isolation procedures

Intact mitochondria and bacteroids were isolated following the procedure of Day et al. (1986). Thirty to 50 g of nodules were homogenized in 300 ml of ice-cold homogenisation buffer (0.4 M sorbitol, 50 mM TES buffer, 2 mM EDTA, 10 mM KH_2PO_4, 30 mM ascorbate, 2% (w/v) PVP-40 and 1% (w/v) BSA). The final pH was adjusted to 7.6 with KOH. The homogenate was filtered and centrifuged in a swing-out rotor at 4000×g for 5 min at 4 °C.

The pellet, containing bacteroids and cell debris, was resuspended in wash medium (0.4 M sorbitol, 10 mM TES buffer pH 7.2 and 1% (w/v) BSA), layered over 30 ml of wash medium containing 70% Percoll (v/v) and centrifuged at 40000×g for 30 min. The purified bacteroids were located in a broad band near the bottom of the tube. This band was removed by suction, diluted at least 5-times in wash medium and the bacteroids pelleted by centrifuging at 10000×g for 10 min. Then the bacteroids were resuspended in wash medium at a concentration of approximately 20 mg protein.ml^{-1} and kept on ice until assayed.

The 4000×g supernatant (above) was carefully decanted and recentrifuged at 10000×g for 15 min. The pellet, which contained mitochondria, plastids, bacteroids and membrane fragments, was resuspended in about 10 ml of wash medium, layered over 30 ml of wash medium containing 45% Percoll (v/v) and centrifuged at 40000×g for 30 min. The mitochondria were located in a tight brown band near the top of the tube, together with plastids, peroxisomes and membrane fragments. This band was removed by suction, diluted at least 5-times with wash medium and concentrated by centrifuging at 15000×g for 10 min. The loose pellet was resuspended in about 5 ml of wash medium and applied to the top of 30 ml of wash medium containing 28% Percoll (v/v) and a linear gradient of 0–10% (w/v) PVP-25 and centrifuged at 40000×g for 30 min. The mitochondria were found in a pale brown band near the bottom of the tube and were washed and concentrated as described above for bacteroids. The mitochondria were finally resuspended in 1–2 ml of wash medium, at a protein concentration of about 10 mg.ml^{-1}.

The degree of purity of the preparations was regularly monitored by electron microscopy (Price et al., 1987)

Oxygen uptake measurements

Oxygen uptake was measured using a Clark-type electrode DW 1 (Hansatech, England) in 2 ml of reaction medium with 0.7 mg mitochondrial or bacteroid protein at 25 °C and pH 7.2. The standard reaction medium contained 0.4 M sorbitol, 5 mM $MgCl_2$, 10 mM phosphate buffer, 10 mM TES buffer pH 7.2 and 1% (w/v) BSA. The concentration of O_2 in air-saturated medium was taken to be 246 μM.

Protein measurement

Protein was estimated by the method of Lowry et al. (1951).

Results

Oxygen uptake by mitochondria

Nodule mitochondria readily oxidized all substrates tested with a good respiratory control. In all our experiments, succinate was used as a substrate for mitochondrial respiration (Fig. 1–6), and for comparison the oxygen uptake with malate as a substrate was presented (Fig. 7). Results presented in Fig. 1, 3 and 5 show that mitochondria isolated from control soybean nodules, and the ones from soybean plants treated with malate and citrate during inoculation, have similar levels of respiratory control ratios (3.6–4.0, 3.9–4.1 and 3.88–4.06, respectively). Higher respiratory control ratios were obvious for mitochondria, isolated from nodules of plants that are treated with succinate and α-ketoglutarate during inoculation (3.57–4.42 and 3.51–4.5 respectively) (Fig. 2 and 4).

Mitochondria isolated from soybean nodules were highly sensitive to the respiratory inhibitor antimycine A. The antimycine-resistant oxygen uptake was 5–10% of the rate of control mitochondrial respiration (Fig. 1–5).

Data presented in Fig. 6 and 7 show significant differences in the rates of oxygen uptake by mitochondria isolated from soybean treated with various organic acids. An increase of mitochondrial oxygen uptake was observed after treatment with succinate, malate and α-ketoglutarate during inoculation. Rates of oxygen consumption were similar to the control values after plant treatment with citrate. Oxygen uptake by nodule mitochondria was influenced to a great extent by the type of substrate used. When succinate was used as an energy-yielding substrate (Fig. 6), mitochondrial respiration was 80% higher than in the case when malate was used (Fig. 7).

The stimulation of oxygen uptake by mitochondria was from 25 to 30% greater when NADH was added together with succinate and malate as substrate than in the case when only these organic acids were applied.

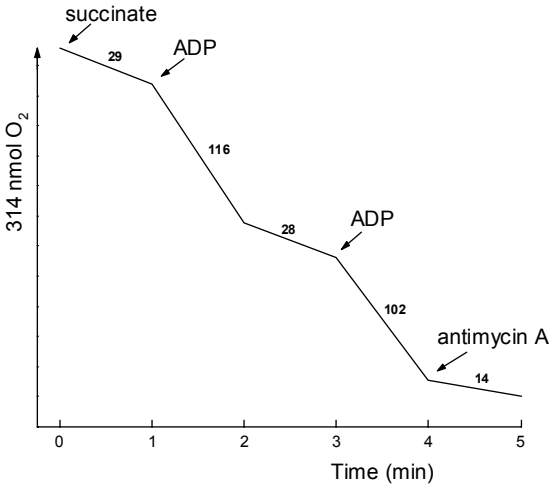

Fig. 1. Oxygen consumption by mitochondria isolated from nodules of soybean plants, grown on Hellriegel's solution (control). In 2 ml of standard reaction medium 10.0 mM succinate, 0.3 mM ADP and 5 μM antimycin A were added as shown. Numbers on traces refer to nmol O_2.min^{-1}.mg^{-1} protein. Each point represents the mean value (±SE) of four replicates.

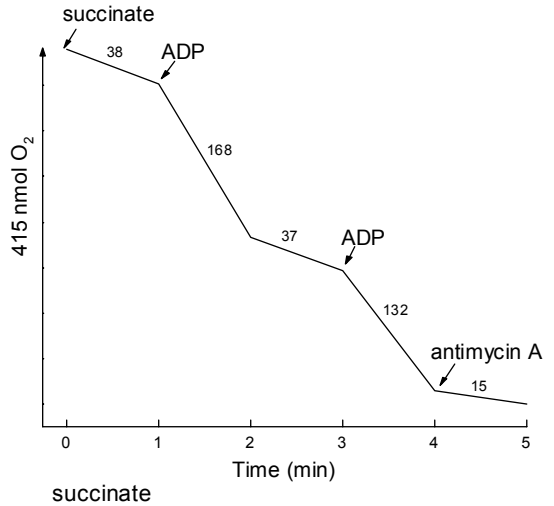

Fig. 2. Oxygen consumption by mitochondria isolated from nodules of soybean plants, grown on Hellriegel's solution and treated with 200 μM succinate during inoculation. Details as in Fig. 1.

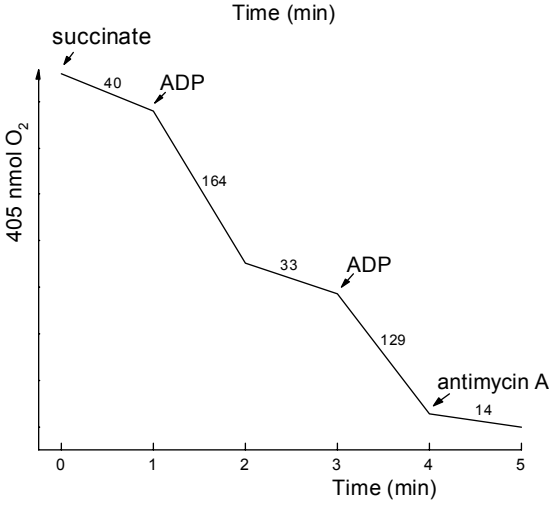

Fig. 3. Oxygen consumption by mitochondria isolated from nodules of soybean plants, grown on Hellriegel's solution and treated with 200 μM malate during inoculation. Details as in Fig. 1.

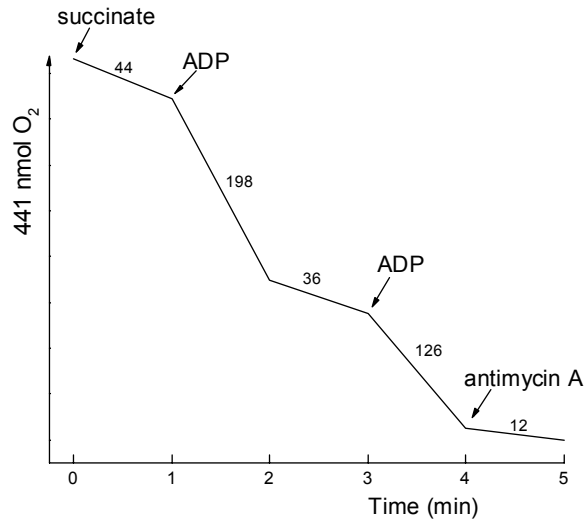

Fig. 4. Oxygen consumption by mitochondria isolated from nodules of soybean plants, grown on Hellriegel's solution and treated with 200 μM α-ketoglutarate during inoculation. Details as in Fig. 1.

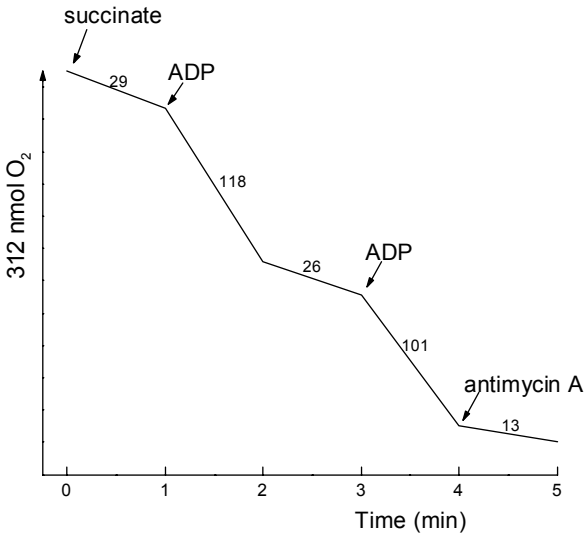

Fig. 5. Oxygen consumption by mitochondria isolated from nodules of soybean plants, grown on Hellriegel's solution and treated with 200 μM citrate during inoculation. Details as in Fig. 1.

Oxygen uptake by bacteroids

Bacteroid respiration was characterized by a significant endogenous rate of oxygen consumption (Fig. 8a and 9a), which could be stimulated by exogenous addition of organic acids (Fig. 8b and 9b). Oxygen uptake with 10 mM succinate was about 20% higher than that with 10 mM malate (Fig. 8b and 9b). Addition of NADH to the incubation medium did not stimulate the rates of bacteroid oxygen uptake (Fig. 8c and 9c) in comparison to the assays with a substrate of only succinate or malate (Fig. 8b

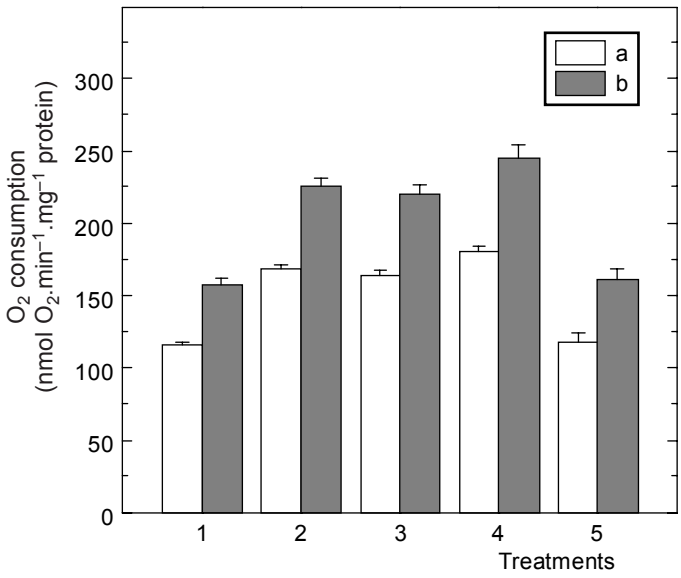

Fig. 6. Oxygen consumption by mitochondria isolated from nodules of control soybean plants (1) and plants treated during inoculation with (2) malate, (3) succinate, (4) α-ketoglutarate, (5) citrate. As exogenous substrate was used: a – 10 mM succinate; b – 10 mM succinate and 1 mM NADH. The mitochondrial respiration was in state 3 (refers to O_2 uptake in the presence of ADP). Each point represents the mean value (±SE) of four replicates.

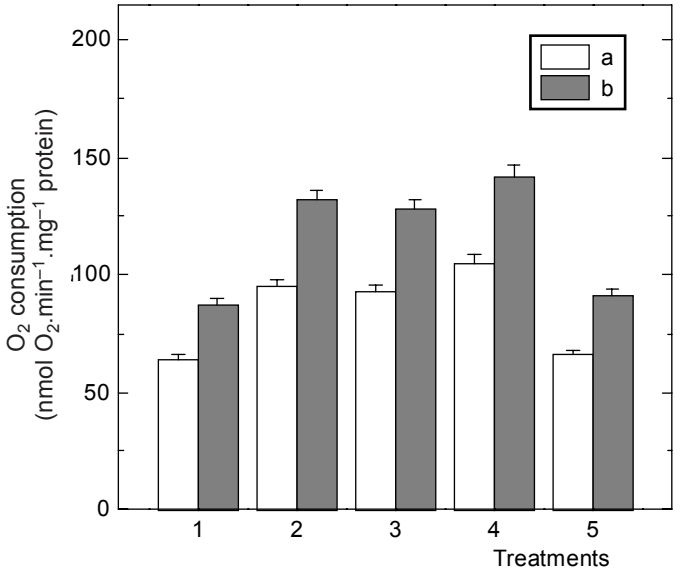

Fig. 7. Oxygen consumption by mitochondria isolated from nodules of control soybean plants (1) and plants treated during inoculation with (2) malate, (3) succinate, (4) α-ketoglutarate, (5) citrate. As exogenous substrate was used: a – 10 mM malate; b – 10 mM malate and 1 mM NADH. The mitochondrial respiration was in state 3 (refers to O_2 uptake in the presence of ADP). Each point represents the mean value (±SE) of four replicates.

and 9b). In some cases, oxygen uptake of bacteroids with succinate and NADH as substrates showed some resemblance of respiratory control of mitochondria, as the addition of ADP stimulated the oxygen uptake with 46 and 31%, respectively (Fig.

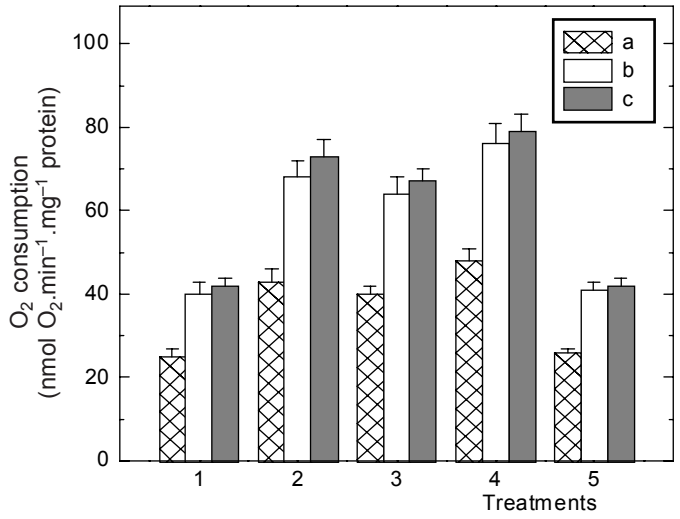

Fig. 8. Oxygen consumption by bacteroids isolated from nodules of control soybean plants (1) and plants treated during inoculation with (2) malate, (3) succinate, (4) α-ketoglutarate, (5) citrate. a – endogenous oxygen consumption; b – with 10 mM succinate; c – with 10 mM succinate and 1 mM NADH. Each point represents the mean value (±SE) of four replicates.

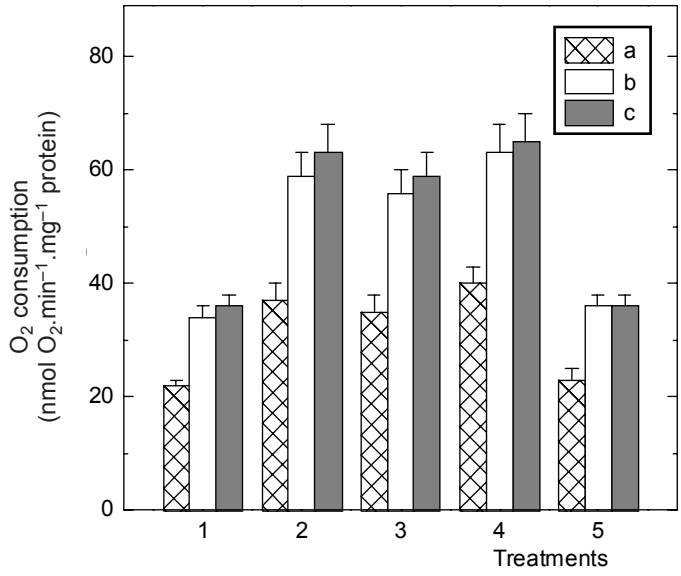

Fig. 9. Oxygen consumption by bacteroids isolated from nodules of control soybean plants (1) and plants treated during inoculation with (2) malate, (3) succinate, (4) α-ketoglutarate, (5) citrate. a – endogenous oxygen consumption; b – with 10 mM malate; c – with 10 mM malate and 1 mM NADH. Each point represents the mean value (±SE) of four replicates.

10). When plants were treated with citrate during the inoculation the oxygen consumption by soybean bacteroids had similar rates with the control ones (Fig. 8 and 9). Higher oxygen uptake was observed for bacteroids from nodules of plants, treated during inoculation with succinate, malate and α-ketoglutarate (Fig. 8 and 9).

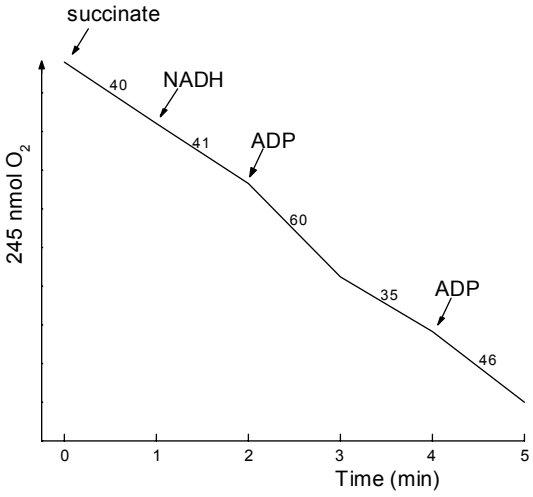

Fig. 10. Oxygen consumption by bacteroids isolated from nodules of soybean plants, grown on Hellriegel's solution (control). In 2 ml of standard reaction medium 10.0 mM succinate, 0.3 mM ADP and 5 µM antimycin A were added as shown. Numbers on traces refer to nmol $O_2.min^{-1}.mg^{-1}$ protein. Each point represents the mean value (±SE) of four replicates.

Discussion

Intact and functional nodule mitochondria largely free from contamination by other membrane components were produced by the method of Day at al. (1986). Electron microscopy studies showed that mitochondria, isolated from soybean nodules, possessed a well defined matrix and largely intact membranes (Day et al., 1986).

Contamination of bacteroid preparations by mitochondria can be ruled out because we observed no measurable stimulation of oxygen uptake upon addition of NADH to the reaction medium (Fig. 8c and 9c), while such NADH oxidation is very rapid in isolated nodule mitochondria (Fig. 6b and 7b). These results are consistent with those of Day et al. (1986).

In the last few years, there have been considerable advances in the characterization of the cyanide-resistant alternative pathway associated with plant respiration. The pathway consists of a single enzyme, a cyanide-resistant quinol oxidase called the alternative oxidase (AO), that shunts electrons off the cyanide-sensitive Cyt pathway at the level of the ubiquinone pool and reduces oxygen to water with no conservation of energy (Wagner and Krab, 1995). Studying the partitioning of electron flow between the alternative and Cyt pathways by use of isolated mitochondria, Bahr and Bonner (1973) found that low concentrations of Cyt pathway inhibitors such as cyanide or antimycin A diverted electrons onto the alternative pathway. In our experiments the non-phosphorylating alternative pathways were detected by their insensitivity to the inhibitor antimycine A. The mitochondria from soybean plant nodules showed high sensitivity to this inhibitor, which means that a non-phosphorylating pathway is absent in the nodules and the energetic effectiveness of these organelles is very high (Fig. 1–5). The latter was proven by the good respiratory control ratios, observed in

nodule mitochondria from soybean plants that were treated with succinate and α-ketoglutarate during inoculation (Fig. 2 and 4).

The insignificant number of branches from the respiratory chain of nodule mitochondria which do not accumulate energy is yet another metabolic divergence that is realized in the differentiation of root cells during nodulation. It is fully consistent with the high energy dependence of the infected nodule cells during fixation of atmospheric nitrogen and the formation of ammonia. These results confirm the data obtained by de Visser and Lambers (1983) who have established that respiration of nodules is significantly more effective than the one of roots.

Free oxygen concentration within infected cells of nodules is about 10 nM and increases to about 20–26 nM at the cell periphery (Sheehy et al., 1985). The location of mitochondria at the periphery of mature infected cells (Bergersen and Goodchild, 1973; Newcomb et al., 1985) is probably related to the need for access to higher oxygen concentrations (Rawsthorne and LaRue, 1985). Nevertheless, these levels of oxygen concentration are apparently sufficient to maintain functional mitochondrial integrity.

Our experiments show that mitochondria in state 3 have high rate of oxygen consumption (in the range of 102 nmol to 198 nmol O_2.min^{-1}.mg^{-1} protein) and probably together with leghaemoglobin maintain a low level of oxygen in the infected cells which creates optimal conditions for nitrogenase functioning. A significant difference in the rate of oxygen uptake by mitochondria isolated from soybean plant nodules was observed in all treatments investigated. The control plants and the ones treated with citrate during inoculation had a lower rate of oxygen consumption, but this rate was higher in the plants treated with succinate, malate and α-ketoglutarate.

Bacteroid respiration was characterized by significant endogenous rate of oxygen uptake which could be reduced by placing the soybean plants in the dark for two–three days prior to bacteroid isolation (Carroll, 1985), presumably because endogenous substrates became depleted when photosynthate supply to the nodule ceased. Our previous investigations (Ignatov and Vassileva, 1999) and results presented in Fig. 8 and 9, fully confirm the dependence of plant photosynthesis and bacteroid respiration. Photosynthetic intensity and the oxygen uptake of bacteroids were lowest in the control soybean plants and the ones treated with citrate during inoculation. In cases when the intensity of photosynthesis was high (treatments with succinate, malate and α-ketoglutarate added) the rate of oxygen uptake of the bacteroids was highest.

In conclusion, after treatment of soybean plants with organic acids succinate and α-ketoglutarate, the oxidative capacity of mitochondria and bacteroids from their root nodules was stimulated. It seems reasonable to assume that this stimulation may lead to a higher nodule nitrogen fixation, that was established in our previous study (Ignatov and Vassileva, 2000).

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References

Bahr, J. T., W. D. Bonner, 1973. Cyanide insensitive respiration. II. Control of the alternative pathway. J. Biol. Chem., 248, 3446–3450.

Bergersen, F. J., D. J. Goodchild, 1973. Aeration pathways in soybean root nodules. Aust. J. Biol. Sci., 26, 729–740.

Carroll, B. J., 1985. The plant contribution to the soybean-*Rhizobium* symbiosis. Ph. D. thesis. Australian National University, Canberra, Australia.

Day, D. A., G. D. Price, P. M. Gresshoff, 1986. Isolation and oxidative properties of mitochondria and bacteroids from soybean root nodules. Protoplasma, 134, 121–129.

De Visser, R., H. Lambers, 1983. Growth and efficiency of root respiration of *Pisum sativum* as dependent on the source of nitrogen. Physiol. Plant., 58, 533–543.

Hellriegel, H., 1898. Die methode der sandkultur. Arb. dtsch. landwirtsch. Ges. 34, 7–19.

Hoagland, D. R., D. I. Arnon, 1950. The water-culture method for growing plants without soil. Calif. Agric. Exp. Stn. Circ., 347, 1–39.

Ignatov, G., V. Vassileva, 1999. Investigation of net-photosynthesis, transpiration and stomatal resistance of treated with malate, succinate, α-ketoglutarate and citrate soybean plants. Compt. rend. Acad. bulg. Sci., 52(7–8), 77–80.

Ignatov, G., V. Vassileva, 2000. Influence of organic acids on the symbiotic characteristics of the *Bradyrhizobium japonicum* strain 273 – *Glycine max* nitrogen-fixing system. Compt. rend. Acad. bulg. Sci., 53(1), 73–76.

Ignatov, G., T. Kovacheva, V. Vassileva**,** 2000. Effect of organic acids on the assimilation of nitrate nitrogen by soybean plants and on the adhesion of *Bradyrhizobium japonicum* to their root surface. Russ. J. Plant Physiol., 47(1), 17–22.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, R. J. Randall, 1951. Protein measurement with the Folin reagent. J. Biol. Chem., 193, 265–275.

Newcomb, E. H., S. R. Tardon, R. R. Kowel, 1985. Ultrastructure specialization for ureide production in uninfected cells of soybean root nodules. Protoplasma, 125, 1–12.

O'Brian, M. R., R. J. Maier, 1989. Molecular aspects of the energetics of nitrogen fixation in *Rhizobium*-legume symbioses. Biochim. Biophys. Acta, 974, 229–246.

Ou Yang, L.-J., M. K. Udvardi, D. A. Day, 1990. Specificity and regulation of the dicarboxylate carrier on the peribacteroid membrane of soybean nodules. Planta, 182, 437–444.

Ou Yang, L.-J., J. Whelan, C. D. Weaver, D. M. Roberts, D. A. Day, 1991. Protein phosphorylation stimulates the rate of malate uptake across the peribacteroid membrane of soybean nodules. FEBS Lett., 293, 188–190.

Price, G. D., D. A. Day, P. M. Gresshoff, 1987. Rapid isolation of intact peribacteroid envelopes from soybean nodules and demonstration of selective permeability to metabolites. J. Plant Physiol., 130, 157–164.

Rawsthorne, S., T. A. LaRue, 1985. Respiration and oxidative phosphorylation of mitochondria from nodules of cowpea (*Vigna unguiculata* L.). In: Nitrogen Fixation Research Progress, Eds. H. J. Evans, P. J. Bottomley and W. E. Newton, Martinus Nijhoff Publishers, Boston, pp. 351–375.

Sheehy, J. E., F. R. Minchin, J. F. Witty, 1985. Control of nitrogen fixation in a legume nodule: an analysis of the role of oxygen diffusion in relation to nodule structure. Ann. Bot., 55, 549–562.

Vincent, J. M., 1970. A Manual for the Practical Study of the Root Nodule Bacteria. IBP Handbook No15, Blackwell Scientific Publications, Oxford.

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