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Improved Method for Isolation of Coupled Mitochondria of *Araucaria angustifolia* (Bert.) O. Kuntze

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ABSTRACT

A method for the isolation of coupled mitochondria from the callus of Araucaria angustifolia *is described for the first time. Mitochondria were isolated from embryogenic callus of* A. angustifolia. *They were metabolically active, able to sustain oxidative phosphorylation as shown by respiratory control ratio values, which were about 2.4 when respiring on succinate as substrate. Oxygen uptake experiments, using freeze-thawed disrupted mitochondria, showed the presence of alternative rotenone-insensitive NAD(P)H dehydrogenases, which were stimulated by* Ca^{2+}. *The procedure now described for the isolation of* A. angustifolia *mitochondria is an important new tool, allowing the investigation of mitochondrial bioenergetics and metabolism and physiology of plants.*

Key words: *Araucaria angustifolia*, callus, plant mitochondria, respiratory chain

INTRODUCTION

Araucaria angustifolia (Bert.) O. Kuntze, known as "Paraná pine" or "Pinheiro do Paraná" is a conifer species belonging to the Araucariaceae family, which is widespread in southern Brazil, especially in the cold highlands of the States of Paraná, Santa Catarina and Rio Grande do Sul (Guerra et al., 2000; Zandavalli et al., 2004). Several studies have been carried out both with the callus and with tissues of *A. angustifolia* at different developmental stages of differentiation (Fonseca et al., 2000; Guerra et al., 2000), including the extraction of flavones from the latter, but mitochondria of *A. angustifolia* have never been isolated before. In contrast to mammals, the respiratory chain in plant mitochondria has been shown to possess at least four alternative rotenone-insensitive NAD(P)H dehydrogenases in the inner mitochondrial membrane for transferring electrons to ubiquinone (Melo et al., 1996; Møller, 2001). These enzymes are non-proton pumping, energetically wasteful, and might avoid the production of reactive oxygen species by preventing over-reduction in the respiratory chain (Melo et al., 2001). Two of these alternative NAD(P)H dehydrogenases are found on the outer surface of the inner mitochondrial membrane, facing the intermembrane space and two on the inner surface facing the matrix, similar to what happens with complex I (EC 1.6.5.3) (Møller, 2001). The two external NAD(P)H and the internal

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NADPH dehydrogenases are Ca^{2+} dependent (Melo et al., 1996). The two external enzymes oxidize the cytosolic pyridine nucleotides and possibly operate *in vivo* mainly under stress conditions, activated by increased cytosolic concentrations of free Ca^{2+} that occurs under such situations. The functions of the two internal enzymes are not known, although it has been hypothesized that the internal, rotenone-insensitive NADH dehydrogenase acts as an overflow mechanism when complex I (EC 1.6.5.3) is overburdened (Møller and Palmer, 1982).

We now report for the first time an efficient procedure for the isolation of mitochondria from the callus of *A. angustifolia* and also demonstrate its functionality by oxidative phosphorylation. The presence of alternative rotenone-insensitive NAD(P)H dehydrogenases is reported as well.

MATERIALS AND METHODS

Plant Material

The embryogenic callus of *A. angustifolia* (Astarita and Guerra, 2000), grown on BM culture medium (Gupta and Pullman, 1991; Santos et al., 2002) supplemented with 2 $mg.L^{-1}$ glycine, 0.5 $mg.L^{-1}$ pyridoxine.HCl, 0.5 $mg.L^{-1}$ nicotinic acid, 1 $mg.L^{-1}$ thiamine.HCl, 500 $mg.L^{-1}$ casein hydrolysate, 100 $mg.L^{-1}$ *myo*-inositol, 1 $g.L^{-1}$ L-glutamine, 30 $g.L^{-1}$ sucrose, 7 $g.L^{-1}$ Phytagar (Gibco®), 2 µM 2,4 dichlorophenoxyacetic acid, 0.5 µM benzylaminopurine and 0.5 µM kinetin, was used as the source of mitochondria. The pH of the culture medium was adjusted to 5.8 with KOH prior to autoclaving at 121ºC for 20 min. Casein hydrolyzate and L-glutamine solutions were filter sterilized and added to the medium after autoclaving.

Oxygen Uptake

Oxygen consumption by isolated mitochondria was measured using a Clark-type electrode (Yellow Springs Instruments) connected to a Gilson oxygraph using a standard reaction medium containing 0.25 M sucrose, 10 mM K-HEPES (pH 7.2), 2 mM KCl, 0.2 g% essentially fat free bovine serum albumin (BSA), 2 mM Pi (NaH_2PO_4), 10 µM rotenone and 2 mM succinate, in a final volume of 1.2 mL at 28ºC. No difference in oxygen uptake was found for mitochondria regardless of the presence or absence of ATP, showing that in our preparations the maximal rate of succinate oxidation was obtained. The respiratory rates are expressed in ng atom $O.min^{-1}.mg^{-1}$, considering the oxygen solubility in water at 28ºC and 1 atm as 233 µM (Estabrook, 1967).

NAD(P)H Oxidation Capacity

The oxidation of NAD(P)H was measured indirectly by oxygen consumption in 1.2 mL of standard reaction medium at 28ºC in the presence of 0.3 $mg.mL^{-1}$ of mitochondrial protein, disrupted by 3 cycles of freeze-thawing in liquid nitrogen in the presence or absence of 1 mM Ca^{2+}. The oxygen consumption was induced by the addition of 2 mM NADH or NADPH. The rotenone-insensitive NAD(P)H oxidation capacity was measured in the presence of 10 µM rotenone. The rotenone-sensitive activity was determined as the difference between oxygen consumption in the presence and absence of rotenone. The NAD(P)H oxidation capacity is expressed in ng atom $O.min^{-1}.mg^{-1}$.

Protein Analysis

Protein concentration were determined by the method of Lowry et al. (1951) using BSA as standard.

Ultrastructural Analysis

Samples (0.2 g) of *A. angustifolia* callus (20 days old), grown in the same culture medium, were fixed by immersion overnight in 4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and submitted to low vacuum during the first 2 h. Post-fixation was carried out in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2 (1 h), and dehydrated in an acetone series. Samples were embedded in Spurr resin. The samples were analyzed using a JEOL JEM-1200 EXII transmission electron microscope. Embedded tissue samples were sectioned and stained with 1% uranyl acetate in absolute ethanol for 20 min and then lead citrate for 5 min.

Chemicals

NADH, NADPH, ADP, EGTA, BSA, rotenone, succinic acid, pyridoxine, nicotinic acid, thiamine, Hepes [N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)], glycine, casein hydrolyzate, *myo*-inositol, L-glutamine and FCCP (carbonyl cyanide 4-trifluoromethoxyphenyl-hydrazone), were purchased from Sigma Chemical Co.

(St. Louis, USA). Phytagar was purchased from Gibco (Rockville, USA). All other reagents were commercial products of the highest purity available.

RESULTS AND DISCUSSION

We now present for the first time an effective procedure for the isolation of functionally intact mitochondria from the callus of *A. angustifolia*. Fig. 1 shows an electron micrograph of a thin *A. angustifolia* callus section in which the abundance of mitochondria can be seen.

Isolation of Mitochondria from the Callus of *Araucaria angustifolia*

In Fig. 2, a scheme showing the procedure used for the isolation of mitochondria from the callus of *A. angustifolia*, is presented. Mitochondria were isolated by conventional differential centrifugation, as previously described for the isolation of potato tuber mitochondria (Beavis and Vercesi, 1992 modified by Fortes et al., 2001) with modifications, as described below. The callus was first cut with scissors, then smoothly homogenized in a van Potter-Elvehjem homogenizer and after that disrupted in a Turratec homogenizer by 4 s bursts in the presence of a cold extraction medium containing 0.25 M sucrose, 3 mM cystein, 2 mM EGTA, 0.2 g% BSA, 10 mM Na-Hepes, pH 7.6, (35 g of fresh callus/200 mL medium). The homogenate was filtered through nylon cloth, and the pH was adjusted to 7.2. The filtrate was centrifuged for 10 min at 1000Xg. The supernatant was centrifuged for 10 min at 15000Xg and each pellet was resuspended in wash medium (0.25 M sucrose, 0.25 mM EGTA, 0.2 g% BSA, 10 mM Na-Hepes, pH 7.2) and transferred to a single tube and centrifuged for 10 min at 1000Xg. The supernatant was centrifuged for 10 min at 15000Xg. Mitochondria, found as a beige coloured pellet, were resuspended in one drop of wash medium and kept on ice until use.

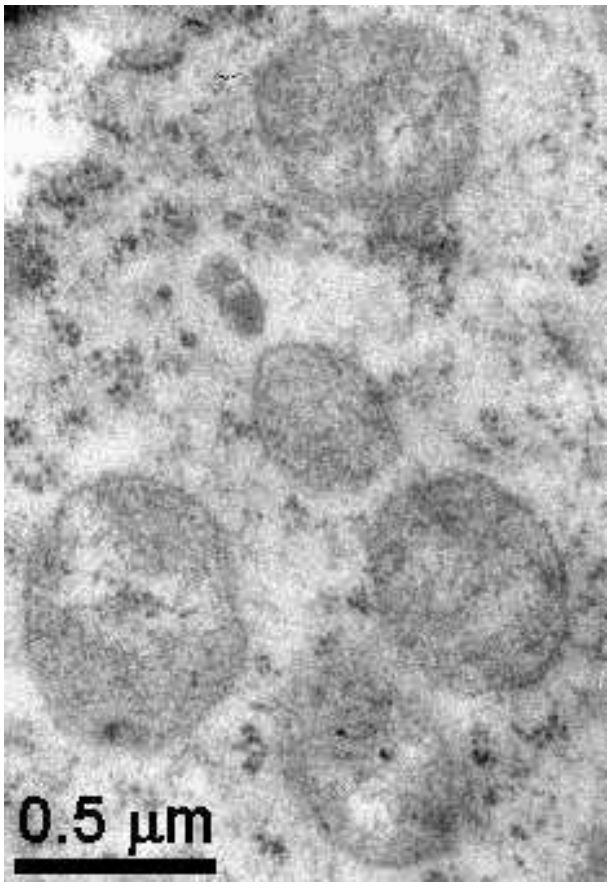

Figure 1 - Electron micrograph of a *A. angustifolia* callus cell (20 days old) showing the abundance of mitochondria.

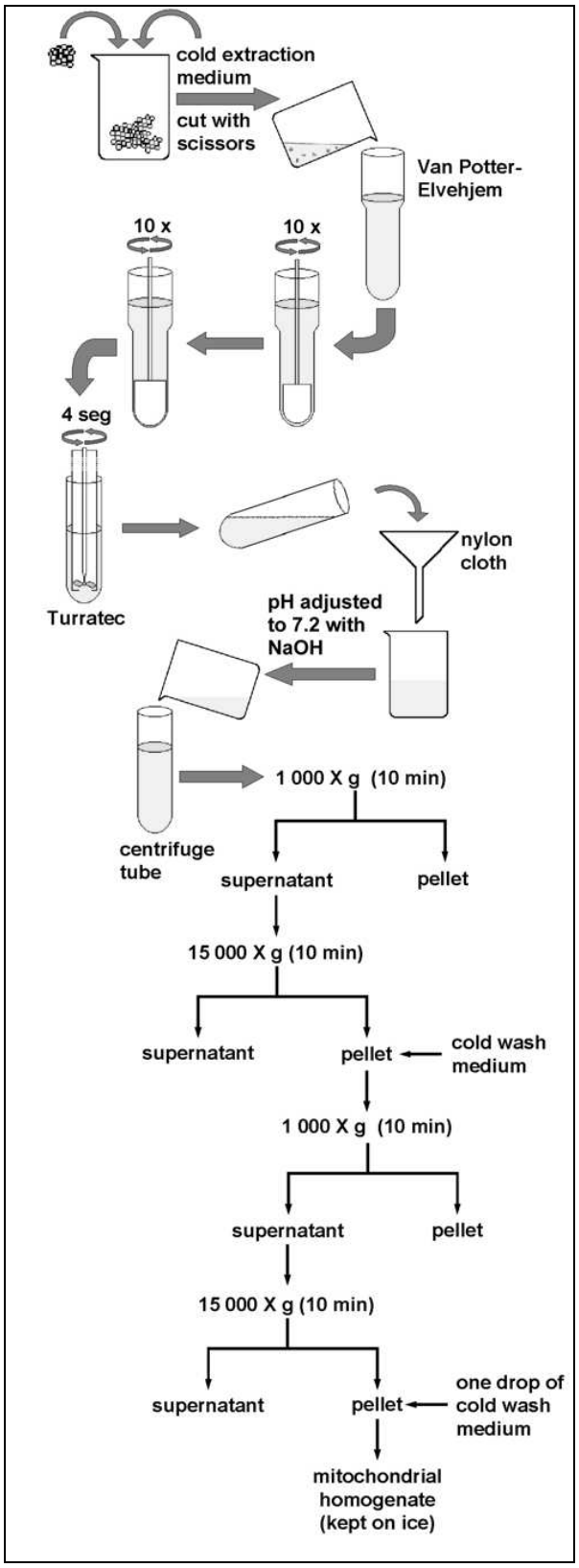

Figure 2 - Scheme demonstrating the procedure used for the isolation of mitochondria from callus of *A. angustifolia.*

Oxygen Uptake by *A. angustifolia* Mitochondria

Fig. 3 shows the oxygen consumption in mitochondria of *A. angustifolia* respiring on succinate in the presence of 1 mM phosphate. By addition of 150 nmol ADP a respiration rate of 78 ng atom.mg^{-1}.mL^{-1} was attained (state 3 respiration) and recovery of the respiration to the resting state (state 4 respiration) of 36 ng atom.mg^{-1}.mL^{-1} was obtained after exhaustion of the added adenine nucleotide.

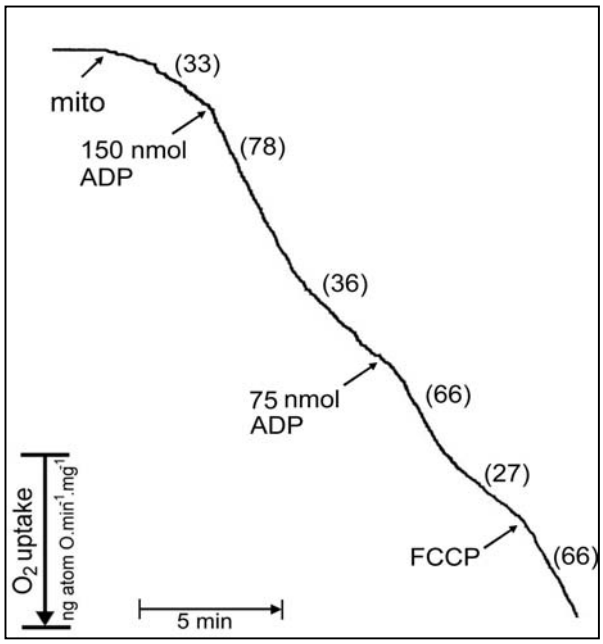

Figure 3 - Measurement of the respiration rate of isolated mitochondria from *Araucaria angustifolia* callus. The reaction medium contained 0.25 M sucrose, 10 mM K-HEPES (pH 7.2), 2 mM KCl, 0.2 g% essentially fat-free BSA, 2 mM Pi, 10 µM rotenone and 2 mM succinate, in a final volume of 1.2 mL, at 28ºC. The O_2 uptake was induced by the addition of 0.3 mg.mL^{-1} mitochondrial protein. Other additions were: 150 and 75 nmol ADP and 1 µM FCCP as indicated. This figure is representative of 6 independent experiments.

There was state 3 and state 4 respiration again after further addition of 75 nmol ADP to the incubation medium denoting the capability of the mitochondria to phosphorylation. The ratio between state 3 and state 4, the respiratory control ratio, is a good index for the integrity of mitochondria. Thus 2.16 and 2.44 are good

respiratory control indexes for plant mitochondria taking into consideration the possible presence of the ubiquitous alternative oxidase (AOX). AOX is an electron transferring oxidase, non-proton pumping, found in all plant mitochondria studied until now, which decreases considerably the respiratory control because of its uncontrolled respiration.

Fig. 3 also shows the increase in the oxygen consumption on addition of the protonophore FCCP, a well known uncoupler of mitochondria which acts causing the collapse of the mitochondrial internal membrane potential, through permeabilization of the membrane to protons.

Alternative NAD(P)H Dehydrogenase and Complex I (EC 1.6.5.3) Activity

Table 1 shows the oxygen consumption rate induced by the oxidation of NADH or NADPH in *A. angustifolia* mitochondria disrupted by freeze-thawing. Mitochondria show rotenone-insensitive NADH and NADPH oxidase activities that were increased by ~18% and 40%, respectively, by the addition of 1 mM Ca^{2+}. On the other hand, the rotenone-sensitive NAD(P)H oxidation was not affected by Ca^{2+} addition. This fact indicates the presence of the alternative NAD(P)H dehydrogenases in *A. angustifolia* mitochondria and also that their activities were modulated by the presence of Ca^{2+}, like the external NAD(P)H and internal NADPH dehydrogenases that occur in potato tuber mitochondria (Melo *et al*, 1996). It could be concluded that complex I (EC 1.6.5.3) in these mitochondria was not affected by the addition of Ca^{2+} since this ion did not affect the rotenone-sensitive activity.

Table 1 - NAD(P)H oxidation capacity in freeze-thawed disrupted *A. angustifolia* mitochondria

	Total activity	Rotenone-insensitive activity	Rotenone-sensitive activity
NADH	54.6 (4.9)	38.5 (1.6)	16.1 (4.3)
+ Ca^{2+}	62.2 (10.2)	45.6 (2.1)	16.6 (8.1)
NADPH	34.5 (1.1)	31.4 (0.5)	3.1 (0.7)
+ Ca^{2+}	46.1 (1.6)	44.0 (0.7)	2.1 (0.9)

- Rates are in ng atom $O.min^{-1}.mg^{-1}$ mitochondrial protein. Each value represents a mean ± SD. Final concentrations were 2 mM NADH, 2 mM NADPH, 1 mM Ca^{2+} and 10 μM rotenone. The plus sign indicates consecutive addition.

CONCLUSIONS

We now report an effective method for the isolation of mitochondria from the callus of *Araucaria angustifolia.* Using this protocol it was possible to measure the oxygen consumption by the mitochondria demonstrating that they were functionally intact and able to sustain oxidative phosphorylation, with a respiratory control of 2.4. The isolated mitochondria were sensitive to the protonophore FCCP, indicating that a transmembrane electrical potential was formed by energization and was sensitive to H^+ permeabilization. *A. angustifolia* mitochondria possess alternative rotenone-insensitive NAD(P)H dehydrogenases, which were stimulated by Ca^{2+}. However, the experiments on freeze-thawed mitochondria (Table 1) did not allow the identification of the alternative NAD(P)H dehydrogenases affected by Ca^{2+} and further studies with intact mitochondria are required.

The study of these alternative dehydrogenases and several other aspects of the respiratory chain of *A. angustifolia* mitochondria are of great importance, especially the study of AOX, as this is a conserved protein along evolution because of its occurrence in fungi, protozoa and plants (Siedow et al., 1995). The Araucariaceae family has an ancient origin in the Triassic Period and the araucarians appear to have maintained a preference for subtropical or mesothermal conditions, illustrated by the present distribution of *A. angustifolia* in southern Brazil (Kershaw and Wagstaff, 2001). A comparison between this ancient gymnosperm and the much more studied angiosperm mitochondria (Beavis and Vercesi, 1992; Fortes et al., 2001; Calegario et al., 2003; Ruy et al., 2004; Camacho et al., 2004) could be of great importance.

This isolation of *A. angustifolia* mitochondria could be an important new tool in studying plants, allowing several investigations on the metabolism

and physiology of the plant and to understand the mechanisms for the maintenance of this species.

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RESUMO

Um procedimento de isolamento de mitocôndrias funcionalmente intactas de calos embriogênicos de *Araucaria angustifolia* foi desenvolvido pela primeira vez em nosso laboratório. Mitocôndrias isoladas por este método são metabolicamente ativas, capazes de sustentar fosforilação oxidativa como mostrado pelo controle respiratório de aproximadamente 2,4, respirando na presença de succinato como substrato. Através de experimentos de consumo de oxigênio com mitocôndrias rompidas em nitrogênio líquido foi demonstrada a presença de NAD(P)H desidrogenases alternativas, insensíveis à rotenona e estimuladas por Ca^{2+}. O isolamento de mitocôndrias de *A. angustifolia* é um novo e importante instrumento para estudar plantas, permitindo a execução de múltiplas investigações a respeito da bioenergética mitocondrial e fisiologia vegetal.

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