**A simply method for the addition of rotenone in *Arabidopsis thaliana* leaves**

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**Abstract**

A simple and reproducible method for the treatment of *Arabidopsis thaliana* leaves with rotenone is presented. Rosette leaves were incubated with rotenone and Triton X-100 for at least 15 h. Treated leaves showed increased expression of *COX19* and *BCS1a*, two genes known to be induced in *Arabidopsis* cell cultures after rotenone treatment. Moreover, rotenone/Triton X-100 incubated leaves presented an inhibition of oxygen uptake. The simplicity of the procedure shows this methodology useful for studying the effect of the addition of rotenone to a photosynthetic tissue *in situ*.

## Introduction

The mitochondrion is the organelle that generates most of the cellular ATP in a process known as oxidative phosphorylation (OXPHOS). This process is catalyzed by five respiratory complexes located in the inner mitochondrial membrane [1]. The complexes protein subunits involved in OXPHOS are encoded by the nuclear and mitochondrial genomes, except for complex II, with subunits all encoded in the nucleus [2].

The NADH:ubiquinone oxidoreductase (called complex I, CI, EC 1.6.99.3), a component of the respiratory chain, is a large multi-subunit complex and has been well characterized in bacteria, fungi, mammals and plants [3-5]. CI contains more than 30 nuclear and mitochondrial-encoded proteins, and genome coordination is required for full activity [6]. At least 14 CI subunits are highly conserved in other eukaryotic and prokaryotic enzymes and a set of 9 proteins widely found in eukaryotic complexes [3]. This complex catalyzes the oxidation of NADH and the subsequent transfer of electrons to ubiquinone, coupled to proton transport across the inner mitochondrial membrane. Therefore, CI would be expected to play a pivotal role in energy production in plant cells [3].

Several compounds such as rotenone, and other insecticides or acaricides had been shown to inhibit CI activity. Mechanistic studies showed that these inhibitors block the electron transport between an iron-sulphur cluster and ubiquinone [7].

The use of knock out mutants or CI inhibitors is important to better understand the role or function of mitochondrial CI. It has been reported that the inhibition of CI activity results in an increase of mitochondrial biogenesis [1, 8]. Furthermore, it has been described that several genes participating in protein import to mitochondria, molecular

chaperones and genes codifying proteins involved in respiratory chain assembly are highly expressed after treatment of *Arabidopsis* cell cultures with rotenone [8]. However, some methodological problems remain to be solved when used rotenone directly on *Arabidopsis* leaves instead of cell cultures. In this work, we present a simple and reproducible method for the addition of rotenone in *Arabidopsis* leaves.

## Materials and Methods

### Plant material and growth conditions

*Arabidopsis thaliana* (var. Columbia Col-0) was used as the wild type. Plants were cultivated on soil in greenhouse conditions at 25°C under fluorescent lamps (Grolux, Sylvania and Cool White, Philips) with an intensity of 150 μmol . $m^{-2}$ . $s^{-1}$ using a 16 h light/8 h dark photoperiod.

### Reagents

Triton X-100 were purchased from Bio-Rad (Hercules, CA, USA); rotenone and TRI reagent were from Sigma-Aldrich (St. Louis, MO, USA)

### RNA isolation, reverse transcriptase (RT)-PCR analysis and blotting

Total RNA was isolated from leaves from 6-week-old plants using the TRI Reagent. cDNA synthesis was accomplished using 3μg of total RNA in the presence of random hexamers (Amersham Biosciences, UK) and MMLV reverse transcriptase (USB Corp. Cleveland, OH, USA) according to the manufacturers' protocol in a total volume of 25 μl. An aliquot (1μl) of the cDNA obtained from RT reaction was used as template in PCR reactions with specific oligonucleotides. Semi-quantitative RT-PCR analysis was performed on the amplification of products after 16, 20, 24, 28 and 30 PCR cycles. Appropriate number of cycles was determined for each cDNA to obtain data during the exponential phase of the PCR reaction. The following primers were used to amplify the desired genes: BCS1up, ATGGAAGGATCCAAGCTAC; BCS1do, CGTGGAGCGTGACGAAGAA; COXup, GGCAAGCTTTCTTTTGTCAACCTTTCA; COXdo, GGCAAGCTTTACAAAGATGATCACTATAAAGTTCG. After electrophoresis on

1.5% agarose gels, the PCR products were transferred onto Hybond N+ membranes (Amersham Biosciences, UK). Probe labeling and membrane hybridization were performed according to the ECL Direct Nucleic Acid Labeling and Detection System protocol (Amersham Biosciences, UK).

**Determination of oxygen consumption**

Oxygen consumption was measured at 25°C using an air-tight chamber fitted with a Clark type electrode (Hansatech leaf disc electrode unit, Hansatech, UK). Detached Arabidopsis leaves (200 to 300 mg) were placed in the oxygen electrode chamber. Calibration was achieved by a simple 2-point calibration between air (21% $O_2$) and the injection or removal of a known volume of air from the chamber. Zero oxygen was achieved by equilibration with $N_2$ to displace all the $O_2$ present in the chamber. Oxygen concentration was monitored for 20 min.

**Rotenone treatment**

Rotenone stock solutions were made by preparing a 1 mM rotenone in a 50:50 solution of 1% V/V methanol:ethylenglycol. Rotenone working solutions were made by diluting the stock solution to 40 µM rotenone in 1 % V/V 50:50 methanol:ethylenglycol. About 250 mg of *Arabidopsis* rosette leaves was used for each experiment. Detached leaves were incubated in 2 ml of a solution containing 40 µM rotenone (working solution) and 0.005% Triton X-100 for at least 15 h at 25°C. Leaves incubated for the same period in distilled water, 40 µM rotenone or 0.005% Triton X-100 were used as control.

After treatment, we determined mRNA levels of *COX19* and *BCS1a* by RT-PCR. Furthermore, we measured oxygen uptake of the treated leaves using an $O_2$ electrode as described above.

**Discussion**

In the present work, we report a simple procedure for the treatment of *Arabidopsis* leaves with the specific mitochondrial CI inhibitor rotenone. The usefulness and reliability of the procedure was determined by the analysis of the expression of two genes involved in respiratory chain assembly, *COX19* (At1g69750) and *BCS1a* (At3g50930). It has been reported an induction in the transcription of *COX19* (1.8-fold) and *BCS1a* (12.2-fold) after rotenone treatment in *Arabidopsis* cell cultures [8]. Indeed, we determined the oxygen consumption in rotenone treated leaves and compared with the control.

The analysis of the transcript levels of *COX19* and *BCS1a* were performed by semi-quantitative RT-PCR after 2 and 15 h of incubation of *Arabidopsis* leaves with water, 40 μM rotenone or 40 μM rotenone plus 0.005 % Triton X-100 (Figure 1). After 15 h of rotenone/Triton X-100 treatment, we found an induction of both transcripts, *COX19* and *BCS1a* (about 3- and 5-fold, respectively). The incubation for longer periods of time results in tissue damage. No variations in *COX19* or *BCS1a* gene expression were found after 2 h of treatment (Figure 1). These results are in good agreement with the induction of these two genes in *Arabidopsis* cell cultures. Moreover, our findings confirm the alteration of nuclear gene expression after a mitochondrial dysfunction described by Lister *et al*, (2004).

In animal mitochondria, rotenone inhibits almost completely the oxidation of mitochondrial NADH by complex I. However, the oxidation of NADH in plant mitochondria is only partially sensitive to rotenone [9]. This is because the existence of additional NADH dehydrogenases involved in NADH oxidation in the plant organelle [10].

To further analyze the usefulness of the proposed method, we also determined the oxygen consumption rate in *Arabidopsis* leaves. Oxygen uptake was compared in control and rotenone/Triton X-100 treated leaves. No significant changes in the respiration rate of *Arabidopsis* leaves were observed after 2 h (data not shown). However, after 15 h of treatment, we found about a 50% decrease in oxygen uptake in leaves incubated with rotenone or rotenone/Triton X-100 (Figure 2). The percentage of inhibition observed for oxygen uptake is in agreement with the 50% of inhibition reported previously in barley and bean [11, 12].

In conclusion, leaves treated with rotenone showed a decreased oxygen uptake without alterations in the transcription of the two genes tested (*COX19* and *BCS1a*), known to be induced after rotenone treatment in cell cultures. The incubation of leaves in the presence of rotenone and Triton X-100 showed decreased oxygen uptake and induction in the transcription of *COX19* and *BCS1a,* in agreement with the results reported by Lister et al. [8].

In summary, the present work describes a simple and reliable procedure for the introduction of rotenone, a specific complex I inhibitor, in *Arabidopsis thaliana* leaves. The procedure presented is suitable for further studies using rotenone directly on leaves.

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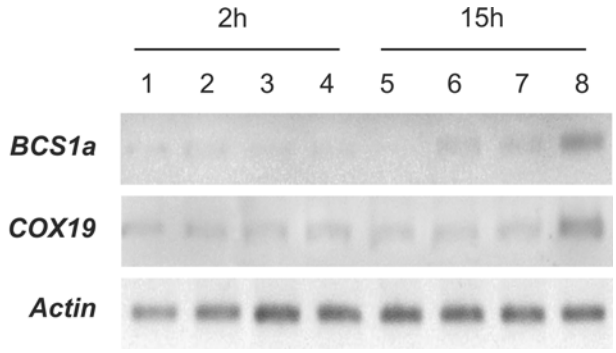
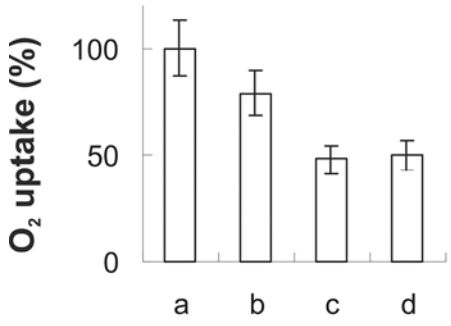
**Figure Legends**

**Figure 1.** Steady state levels of mRNA of *COX19* and *BCS1a* genes in *Arabidopsis* leaves after incubation for 2 or 15 h in different conditions. Lanes 1, 5: water, lane 2, 6: 0.005% Triton, lane 3, 7: 40µM rotenone, lane 4, 8: 40µM rotenone plus 0.005 % Triton. Total RNA was extracted from leaves and reversed transcribed using random hexamers and then amplified using specific primers (see Methods section). The actin gene was used as internal control.

**Figure 2.** Oxygen uptake of *Arabidopsis* leaves after 15 h of treatment in the indicated conditions: a: water, b: 0.005% Triton, c: 40µM rotenone, d: 40µM rotenone plus 0.005 % Triton. 100% correspond to 5.7 µmol $O_2$ . $min^{-1}$. The oxygen consumption was monitored at 25°C for 20 min.


2h
15h
1
2
3
4
5
6
7
8
BCS1a
COX19
Actin


O2 uptake (%)
100
50
0
a
b
c
d