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In situ measurements of oxygen production and consumption using paramagnetic fusinite particles injected into a bean leaf

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Abstract

Photosynthetic oxygen production and respiratory oxygen consumption in the interior of a bean leaf (*Vicia faba*) were monitored by following the amplitude of the electron paramagnetic resonance spectra of coal derivative particles (fusinite) injected into a leaf. We observed the explicit decrease in oxygen concentration due to respiration in the dark, and the light-induced oxygen production (about 2-fold increase in the oxygen partial pressure) only in the case of a closed water-filled chamber which prevented oxygen exchange with the surrounding atmosphere. However, under normal physiological conditions, when the sample was exposed to air (a leaf in the open holder) we did not observe any significant changes in the level of the oxygen partial pressure inside the leaf, neither in the dark nor during illumination. This observation, together with our earlier measurements of oxygen concentration in the aqueous phase of the leaf interior with the microscopic spin-label probes (Ligeza, A., Wisniewska, A. Subczynski, W.K., and Tikhonov, A.N. (1994) Biochim. Biophys. Acta 1186, 201–208), has led us to the conclusion that the ventilation of the leaf interior appears to be sufficient for maintaining the oxygen partial pressure practically on the same level, both in the dark and in the course of intensive light-induced production of oxygen by chloroplasts. Such ventilation should protect leaf tissues against the dangerous increase in the level of oxygen evolved by chloroplasts.

Keywords: Oximetry; Oxygen production; Photosynthesis; Leaf; EPR; (*Vicia faba*)

1. Introduction

Oxygen plays a crucial role in cellular metabolism. The interaction of oxygen with the electron transport chain, intracellular metabolites, and unsaturated lipids results in the formation of active forms of oxygen (the superoxide anion radical, hydrogen peroxide, and hydroxyl radical) which can mediate oxidative injuries in biological tissues (see [1–5]). In plant cells, chloroplasts and mitochondria are the main organelles responsible for the production and consumption of oxygen. The incomplete reduction of oxygen by the chloroplast and mitochondria electron transport chains leads to the formation of superoxide radicals and hydrogen peroxide [6–8]. In chloroplasts, the interaction of molecular oxygen with the triplet state photoreaction centers results in the production of highly reactive singlet oxygen [9] which is responsible for the photodestruction of pigments and degradation of chloroplast proteins [10–13]. The in-

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crease in the concentration of oxygen, as the result of chloroplast functioning, should stimulate the formation of these active forms of oxygen, and thus could provoke the substantial injuries in the energy-transducing apparatus of these organelles. This is a well-documented fact in the case of chloroplasts and mitochondria functioning in vitro [10–13].

However, the question was still open as to whether oxygen produced by chloroplasts in plant cells can be accumulated inside the leaf to a high enough level. To our knowledge, there is a lack of experimental data concerning light-induced changes in the concentration or partial pressure of oxygen in different compartments of the leaf interior, i.e., in the native surroundings of chloroplasts and mitochondria. Electron paramagnetic resonance (EPR) spectroscopy of molecular and microscopic oxygen-sensitive probes [14–19] provides the unique possibility to measure oxygen concentration directly in the leaf interior, and thus to answer the following question: Is the illumination of a leaf accompanied by the increase in oxygen concentration up to the level that might promote destructive processes?

A new method for monitoring oxygen in biological tissues has been suggested recently [20,21]. This method is based on the use of small solid-state paramagnetic particles (e.g., lithium phthalocyanine [20], or a derivative of coal termed fusinite [21]), the EPR spectra of which depend on oxygen concentration. In this work, we have used small fusinite particles injected into bean leaves in order to monitor oxygen production and consumption by chloroplasts and mitochondria in situ. The main advantages of this approach are:

(i) the high sensitivity of the EPR spectrum (the narrow singlet line) given by coal particles to the changes in oxygen partial pressure;

(ii) the chemical and structural stability of this oxygen probe (paramagnetic fragments in the bulk of a coal particle are well-protected from the redox transformations and other side effects, such as the influence of paramagnetic metal ions or pH);

(iii) the lack of toxic effects of coal particles on biological tissues.

In this report, we present results of measurements of oxygen partial pressure inside the leaf that, together with our earlier observations [22], have led us to the conclusion that under normal physiological conditions the ventilation of the leaf interior appears to be sufficient for maintaining the oxygen partial pressure practically at the same level, both in the dark and in the course of intensive light-induced production of oxygen by chloroplasts. Such ventilation should protect leaf tissues against the dangerous increase in the level of oxygen evolved by chloroplasts. This paper complements the work of Belkin et al. [14] in which they analyze oxygen production and consumption by cyanobacteria in a water environment.

2. Materials and methods

2.1. Reagents

Oxygen-sensitive fusinite particles (ground to the size of 1–5 μm) were purchased from Illinois EPR Research Center and used as the aqueous suspension.

2.2. Sample preparation

The leaves were taken from 2-week-old bean plants (*Vicia faba*) grown in soil at room temperature under continuous light illumination. Using a micro syringe, we injected about 0.1 ml of the aqueous suspension of fusinite particles (30 mg/ml) into the leaf midrib. The fusinite particles were suspended either in distilled water or in an isotonic water solution (100 mM KCl). The penetration of the fusinite suspension from the midrib to the leaf tissue was visualized as a black spot expanding around the point of injection. EPR measurements with fusinite-injected leaves were carried out usually 24 h after the injection procedures, so the excess of water filling the intercellular compartments of a leaf had enough time to evaporate. After such a treatment, leaves retained normal turgor without any visible necrosis.

For the EPR measurements, a control or fusinite-injected leaf was cut off the plant, and a 4 mm × 5 mm sample located away from the midrib was cut from the leaf. Samples were placed in one of two different holders. One of the holders, an EPR quartz tissue cell with a depth of 0.6 mm and a glass cover plate, was designed as a closed chamber which isolated the sample from the surrounding atmosphere. Free space inside the chamber was filled with water

to enhance the response of the EPR signal to oxygen production or consumption. Another holder was an open plastic frame which allowed the immediate contact of the leaf surface with surrounding air. All the preparations with leaves before the EPR measurements were performed in the dark or in dim light.

2.3. EPR measurements

The EPR spectra were taken with the Varian E-3 spectrometer at room temperature. To measure the kinetics of oxygen production and consumption, the magnetic field of the EPR spectrometer was fixed at the low-field extreme of fusinite signal. Samples were illuminated with continuous white light (100 W tungsten lamp) inside a rectangle TE102 cavity through an optical window on the front wall. The intensity of light focused on the sample inside the cavity was about 160 W/m^2. The infrared portion of the spectrum was cut off using a 3 cm water filter. Prior to illumination, samples were adapted to the dark for 30 min.

3. Results and discussion

Fig. 1 shows the EPR spectra of a dry fusinite in the air and nitrogen atmospheres. The broadening of the spectral line in the presence of oxygen is caused by spin–exchange interactions between paramagnetic molecules of fusinite and oxygen. The removal of oxygen leads to the narrowing of the line and to the corresponding increase in its amplitude. Fig. 2 demonstrates how the amplitude of the EPR signal of fusinite depends on the pressure of oxygen. We used this dependence as the calibration curve for the quantitative measurements of oxygen concentration inside the leaves.

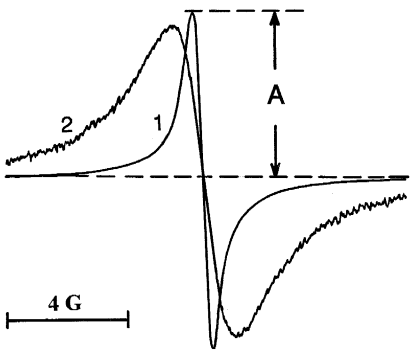

Fig. 1. EPR spectra of dry fusinite particles: 1, in the atmosphere of pure argon; 2, in the atmosphere of pure oxygen. EPR spectra were recorded at the microwave power 10 mW and modulation amplitude 0.5 G. The receiver gain for spectrum 2 was 12.5 times higher than for spectrum 1. *A*, the measured amplitude of the EPR spectrum.

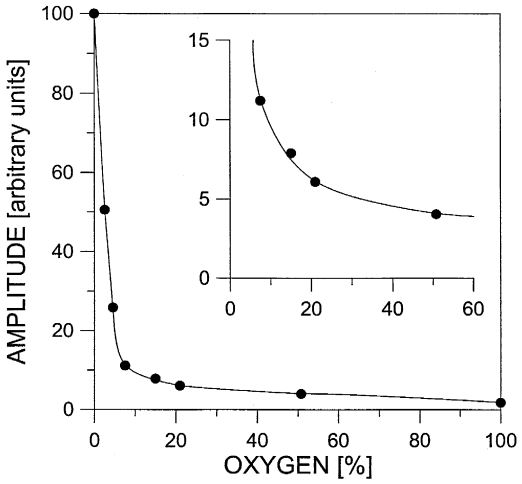

Fig. 2. The amplitude of the EPR spectra of fusinite particles versus concentration of oxygen. Fusinite particles were placed in water equilibrated with different mixtures of pure argon and oxygen adjusted with flow meters. Insert shows relationship with expanded *Y*-axis and the oxygen concentrations that are of most interest for investigated systems.

Fig. 3 demonstrates light-induced changes in the magnitude of the EPR signal from the fusinite particles injected into a bean leaf (curves A and C) or placed on the surface of the control sample of a bean leaf (without injected fusinite, curve B). If the samples were isolated from the surrounding atmosphere (Fig. 3A,B), and, therefore, oxygen could accumulate inside the leaf and water-filled closed chamber, we observed two reversible processes: (a) the oxygen consumption in the dark (the increase in the magnitude of EPR signal) and (b) oxygen production during illumination (the decrease in the magnitude of EPR signal which corresponds to a 2-fold increase in the concentration of oxygen after 5 min of illumination). For dark-adapted leaves, the production of oxygen in response to the first turn of leaf illumination (curve 1) becomes visible only after the lag-phase (about 2 min), while the second switching-on of the

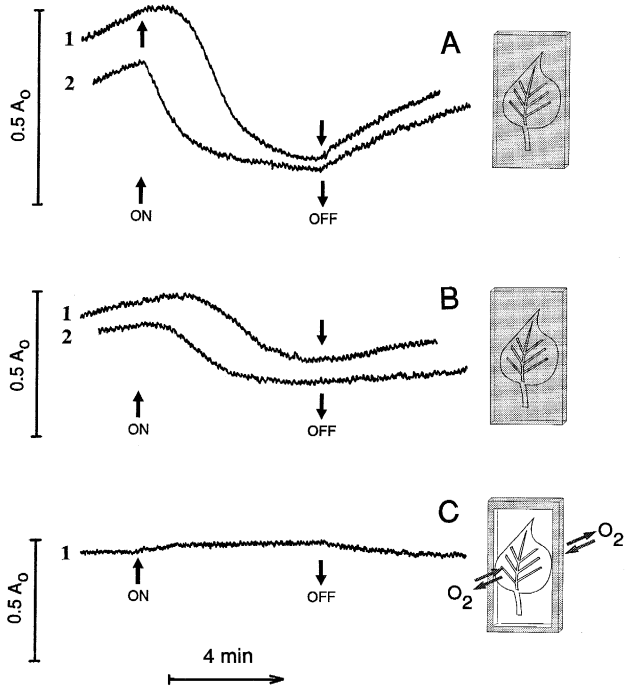

Fig. 3. The typical patterns of the time-courses of the light-induced changes in the amplitude of the EPR signal from fusinite particles injected into a leaf (A,C) or placed on the leaf surface (B). Curves 1 and 2 correspond to the first and the second cycles of illumination, respectively. Before the first illumination, a sample was adapted to the dark for about 30 min. Each curve 2 is the continuation of the corresponding curve 1 (for more details see text). A_0, the amplitude of the EPR spectrum of fusinite injected into the leaf or placed on the leaf surface and equilibrated with air. On the right the sketch drawing shows the leaf (4 mm × 5 mm piece) in the closed chamber or in the open holder.

light (curve 2) results in the immediate production of oxygen. This difference reflects the well-known [23–26] regulatory phenomenon of the light-induced transition of photosynthetic apparatus in intact leaves from State 1 (dark-adapted leaf) to State 2 (a leaf after illumination).

The comparison of Fig. 3A (fusinite inside the leaf) and Fig. 3B (fusinite outside the leaf) demonstrates that the injection of a coal suspension into the leaf does not disturb the ability of a leaf to consume and produce oxygen. Indeed, we can see that the fusinite-injected leaf (Fig. 3A) reveals the same relative changes in the magnitude of the EPR signal as those observed in the control sample (Fig. 3B). This similarity proves that the injection of a fusinite suspension does not inhibit the metabolic activity of plant cell mitochondria and chloroplasts. In this study, oxygen transport across the cut edges of the leaf has been neglected. The rationale for this assumption is 2-fold: (1) the ratio of en face leaf area to area of the edges is very large; and (2) the structure of the leaf is specifically organized to favor transport perpendicular to the surface.

We were able to observe the explicit changes in the oxygen concentration only in the case of a closed water-filled chamber which prevented oxygen exchange with the surrounding atmosphere (Fig. 3A,B). However, when the sample was exposed to air (Fig. 3C, open holder) we did not observe any significant changes in the level of the EPR signal from fusinite inside the leaf, neither in the dark nor during illumination. The EPR measurements presented in Fig. 3C were performed 24 h after the fusinite injection, so the excess of water injected into the intercellular compartments of a leaf had enough time to evaporate. On the other hand, if EPR measurements were performed immediately after the injection of the fusinite suspension (i.e., before the excess of injected water had time to evaporate), we were able to record the light-induced accumulation of oxygen inside the leaf (data not shown). In the latter case, contrary to normal physiological conditions, the intercellular compartments were filled with water, which imposes a substantial barrier to oxygen diffusion. The diffusion coefficient for oxygen in water is substantially smaller (about 10,000 times) than in air. Otherwise, under the normal physiological conditions (Fig. 3C), the light-induced production of oxygen by chloroplasts does not lead to any significant increase in oxygen concentration in the leaf interior. In this case, intensive production of oxygen by chloroplasts is compensated by the fast efflux of oxygen from the leaf to the surrounding atmosphere.

Such a good ventilation of a leaf interior can be explained by rapid, long-distance diffusion of oxygen from chloroplasts to leaf stomata. It has been demonstrated earlier [15,16,27,28], by the use of the conventional oximetric methods and spin-label oximetry, that oxygen is characterized by the high rate of diffusion in lipid bilayers and plasma membranes of different mammalian cells. We have obtained similar results for the thylakoid membranes of chloroplasts [29]. Thus, lipid bilayer membranes are not the barriers to oxygen diffusion from the chloroplast. Under

normal physiological conditions, the long-range transportation of oxygen to the stomata occurs through the water-free intercellular compartments of a leaf interior. The coefficient of oxygen diffusion in air is very high (0.178 cm^2/s at 0°C), and, therefore, the long-range travel of oxygen from oxygen-producing cells to stomata does not retard the leaf ventilation. This conclusion derives additional support from our earlier observation [22]. Using the microscopic water-soluble probe for oxygen (serum albumin-coated light paraffin oil particles containing cholestane spin label), we have demonstrated that the intensive actinic light induces only a very small net increase (less than 2.5%) in oxygen concentration in the bulk of aqueous phase of the leaf interior.

In summary, we conclude that the high rate of the ventilation of a leaf interior prevents the substantial increase in oxygen concentration that might provoke injuries in the energy-transducing apparatus of a leaf.

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