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Effect of *Azospirillum brasilense* and *Burkholderia unamae* Bacteria on Maize Photosynthetic Activity Evaluated Using the Photoacoustic Technique

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Abstract In this work, the photosynthetic process of maize plants (*Zea mays*), which were grown using seeds inoculated with plant growth promoting bacteria *Azospirillum brasilense* and *Burkholderia unamae*, was monitored. Photothermal and photobaric signals obtained by a time-resolved photoacoustic measurement configuration were used for measuring the oxygen evolution rate *in situ*. A frequency-resolved configuration of the method was utilized to determine the oxygen diffusion coefficient and the thermal diffusivity of the maize leaves. The latter parameters, which can be used as indicators of the photosynthetic activity of maize, are found to vary according to the plant–microbe interaction. Treatment with plant growth promoting bacteria induced a decrease in the oxygen diffusion coefficient of about 20 %.

Keywords Biofertilizers · Oxygen evolution rate · Photoacoustic · *Zea mays*

1 Introduction

Conventional agricultural practices cause ecological disruptions, thus contributing significantly to the current climate change. Particularly in regions producing maize (*Zea mays*), the use of monoculture over large territories is not sustainable [1]. Therefore,

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high demands for maize as a food source require the implementation of environmentally friendly strategies of plant nutrition and pest control. One of these strategies is the use of benign microorganisms living in roots, which convert nutritionally important elements available to the plant. The symbiotic interaction has great potential to enhance the degree of nutrient absorption, thus reducing the leaching of nitrate to groundwater. Gaining deeper insight into the interaction among the microbes and plants is crucial to develop effective techniques of growth.

Symbiosis between terrestrial plants and soil microorganisms has become a subject of great scientific interest, as during the past years some devastating side effects of conventional agricultural practices have become clear. The so-called eutrophication process is believed to be the most important cause of 245 000 km^2 of dead zones in the coastal zones of oceans [2]. The use of bacterial inoculants offers a potential alternative for agricultural production sustainability without the use of chemical products [3]. These so-called biofertilizers are produced with benign microorganisms that colonize roots. The strains can be isolated and cultivated under special conditions. The specific use of this kind of bacteria ensures adequate nutrient uptake for plants. This serves for controlling pest and sicknesses that affect their growth and helps to restore the permeability and fertility of super-exploited soils [4]. It does not compromise human health, and the involved economic and environmental costs are low [5,6].

Although plant growth promoting bacteria (PGPB) have been well characterized biologically, further study to evaluate their effects on plants *in situ* is desirable. Effects of *Azospirillum*—plant associations—have been observed by other authors, in particular with respect to the contribution of biological nitrogen (N) fixation to the plant through physiological response examination with the same kind of seeds and inoculation method used in this work. Morphological and physiological changes of the inoculated plant roots with this type of bacterium lead to an enhancement of water and mineral uptake [7]. In addition, nitrogen-fixing *Burkholderia* species have shown a great potential for agricultural applications as a plant growth promoting rhizobacteria, in rhizoremediation, phytoremediation, and pathogens control [8,9]. However, the effects of these specific bacteria strains on maize plant photosynthetic activity are not well known [10].

PGPB improve the plant physiological status (mineral nutrition, water availability, photosynthesis, etc.). The study of the physiological effects of microbial fertilizers is of great importance for agricultural practices and for assessing the productivity of economically important crops, such as maize, after treatment with biofertilizers and N fertilizers. Root inoculation with benign bacteria is certainly a relevant and tested technique for enhancing crop yield. Fuentes-Ramirez and Caballero-Mellado [11] and Dobbelaere et al. [12] have reported evidence for the positive effect of bacterial inoculums on plants, including maize physiology. In these works, the effectiveness of nitrogen-fixing bacteria on the plant production was determined, and the analysis on the plant physiological status (e.g., its mineral nutrition, in particular N, biomass production, chlorophyll content and some morphological parameters) was given. Because photosynthesis and respiration largely determine plant productivity, the focus of this article lies on the assessment of the effects of microbial fertilizers during photosynthetic measurements on maize leaves. The thermal diffusivity of leaves, which is sensitive to the structure and composition, is used as probing quantity.

In order to study the photosynthesis, most authors have used conventional techniques such as manometers, electrochemical sensors, carbon dioxide isotopes, fluorescence and gas exchange methods. Infrared sensors have also been frequently applied for CO_2 measurement, but the approach has the handicap that it limits the amount of time that is required for measuring consumed carbon dioxide [13]. The photoacoustic (PA) technique has been used as alternative [14,15]. It is based mainly on the detection by a microphone in a closed cell of photothermally (PT) induced pressure oscillations in a thin layer of gas adjacent to the surface of the sample, which is illuminated with modulated light and thus dynamically heated [16]. If, in addition, the sample presents photochemical activity, the production of reaction gases contributes to the so-called photobaric (PB) pressure oscillations. In the case of green plant samples, one can study their photosynthetic activity by using time-resolved PA measurements [17,18] that directly sense oxygen release without any gas flux control. Most of the time, the latter mechanism is related to the emission of photosynthetic oxygen. When the photosynthetic process is saturated, then the photobaric signal contribution is quenched, while the photothermal part of the signal remains unaltered. The amplitude ratio between the two contributions, the so-called oxygen evolution rate (OER), is representative for the relative quantum yield of oxygen production.

In this work, the effect of bacterial symbiosis on the photosynthetic process of maize plants has been examined *in vivo* and *in situ* using PA-based OER measurements for*Azospirillum brasilense* and *Burkholderia unamae* bacteria. Several works have reported on the study of the influence of mycorrhiza on photosynthesis in plants, such as tomato and maize, using the so-called open PA cell (OPC) technique [19–21], which uses the front air chamber of an electret microphone as a PA chamber. Here, we have made use of a different PA cell design, which avoids the photothermal contribution from the microphone-metalized electret diaphragm used in OPC, when light transmitted through the leaf is absorbed by it [22].

2 Experimental

Zea mays seeds were inoculated before sowing with two kinds of free nitrogen-fixing bacteria, *Azospirillum brasilense* (strain Cd) and *Burkholderia unamae* (strain MT1-641^T), using the procedures described in detail by Day et al. [23] and by Caballero-Mellado et al. [8], respectively. *A. brasilense* and *B. unamae* were grown during 18 hours in nitrogen-free base (Nfb) and BSE solution (0.5 % succinate, 0.04 % K_2HPO_4, 0.04 % K_2HPO_4, 0.02 % $MgSO_4{\cdot}7H_2O$, 0.05 % yeast extract; pH 6.5, in that order). The seeds were inoculated with 1 mL of this suspension, which was adjusted to an optical density of 0.3 for the first solution and 0.2 for the second one. The seeds were sown in an inert substrate of sterilized river sand, which was placed in 500 mL plastic pots under greenhouse conditions (mean temperature of 16 °C and 70 % of relative humidity).

Photosynthesis analysis was done on 15 plants of maize grown from these seeds. The plants were grouped according to three treatments using two sets of five seedlings inoculated with *A. brasilense* and *B. unamae*, respectively, and an untreated control group. In all cases, 100 mL of the fertilizer in solution ($4\,g{\cdot}L^{-1}$) per week was added to

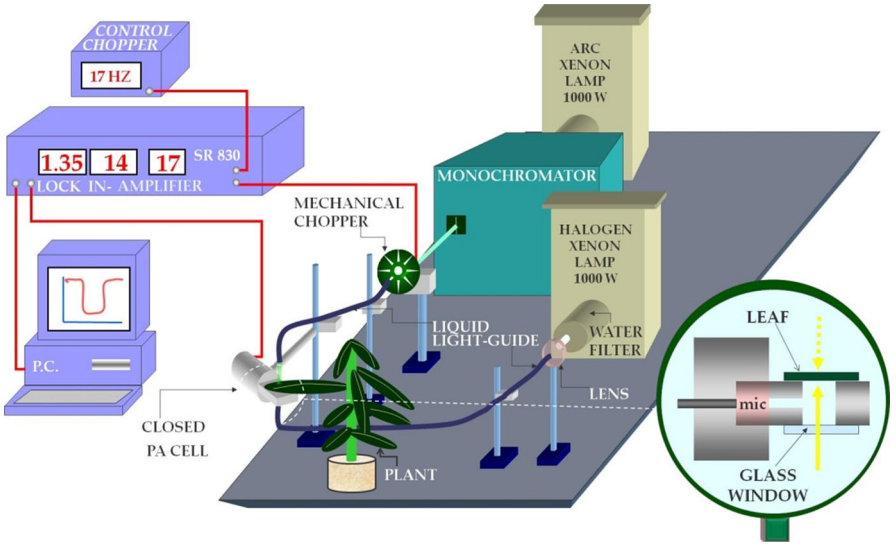

Fig. 1 Scheme of the PA system used for OER measurements showing details of the PA cell and sample's mounting configuration. The leaf closing the PA cell is shown in the *inset*

each sample in the same proportion. Measurements were taken daily in three plants of each group for 6 weeks during summer season after 20 days of sowing. The temperature during the experiments was 23 °C. Measurements with a micrometer gauge (Mitutoyo 547−400) with a resolution of 1 μm yielded an average leaf thickness of (200±5) μm.

For the measurement of the photosynthetic activity, we used a homemade PA spectroscopy system shown schematically in Fig. 1. A 500 W Xenon arc lamp (ORIEL 66924−1000 W) provided white light. A monochromator (ORIEL Cornerstone 130 1/8 m) collimated the light using internal parabolic mirrors and the zero-order spectrum produced by a diffraction grating. A mechanical chopper (Oriel 75159) was used to periodically modulate the light beam intensity at a given frequency. A liquid light guide (Oriel LLG212) directed the collimated and modulated beam (intensity 5 m·W·cm^{-2}) through a transparent window that closed the PA cell onto the leaf sample that hermetically closed a conventional PA cell by means of a natural adhesive. A water filter was introduced to absorb the infrared portion of the lamp spectrum, thus minimizing undesirable heating. An electret microphone coupled to the cell was used to sense the PA signal. The microphone signal was analyzed by a lock-in amplifier (SR850). The whole system was controlled by a personal computer that allows automatic data recording and processing.

Photosynthesis was found to saturate when the plant is exposed to a non-modulated white light beam from a 400 W Xenon halogen lamp (ORIEL 66925−1000 W). The inhibitory effect is caused by light-induced closure of the reaction centers of the leaf and was used *ad hoc* to selectively allow the photothermal signal contribution.

The resulting PA signal, S, was generated by pressure changes in the cell due to both the PT (which included the photochemical loss contribution by energy storage due to carbon fixation [24]) and the PB contribution ($S = PT + PB$). We define

$S' = PT$ the PA signal obtained when saturating the PB effect. Note that although the addition of a non-modulated white light of high intensity saturated the modulated photosynthetic process, it did generate a constant value of the oxygen evolution. Thus, for each sample, the signal amplitude was measured, and a measure for the OER was obtained by considering the porcentual decrease in the signal amplitudes (namely A and A' for the amplitudes of S and S', respectively) when light was turned on, i.e.,

$$\mathrm{OER} = \frac{A - A'}{A} = 1 - \frac{A'}{A}. \tag{1}$$

The value of OER was affected by non-photosynthetic factors, such as the leaf internal anatomy, which influences the diffusion of oxygen through leaf tissues [25]. In order to consider these effects, we followed the methodology described by Poulet et al. [26]. These authors showed that for a modulation frequency range, where an optically opaque sample becomes thermally thick (the sample's thickness, d, is much larger than the so-called thermal diffusion length defined as $(D_{\mathrm{TH}}/\pi f)^{1/2}$, where D_{TH} is the sample's thermal diffusivity), it is also possible to write

$$R = \frac{A - A'}{A'} = R_0 \exp\left[-\pi^{1/2}\left(\frac{1}{D_0^{1/2}} - \frac{1}{D_{TH}^{1/2}}\right) l\, f^{1/2}\right], \tag{2}$$

where R_0 is a detection system-dependent factor, f is the light modulation frequency, l is the average diffusion path length of heat and oxygen from the chloroplast to the boundary of the cellule and D_0 is the mass diffusion coefficient of oxygen in the aqueous medium of the cell, or, for short, the oxygen diffusion coefficient. From measurements of R as a function of the modulation frequency, the oxygen evolution rate can be obtained straightforwardly in case the leaf's thermal diffusivity has been previously determined. The latter can be done as follows: According to the Rosencwaig–Gersho (RG) model [27] (under oxygen evolution saturation conditions for which the PA signal is only due to the photothermal effect), it is well known that for an optically opaque and thermally thick sample the PA signal amplitude is expressed as [28]:

$$\log(A' f) = -d\left(\frac{\pi f}{D_{TH}}\right)^{1/2}, \tag{3}$$

where d is the leaf thickness. Therefore, from the slope of a semi-logarithmic plot of the product $A' \times f$ as a function of $f^{1/2}$ the thermal diffusivity can be directly calculated if d is well known.

3 Results and Discussion

Figure 2 shows typical PA data for the time dependence of the signal amplitude, obtained from leaves of maize plants grown with the different treatments. The modulation frequency was varied between 10 and 37 Hz. At each frequency, when the back non-modulated white light was turned on, the total signal amplitude decreased

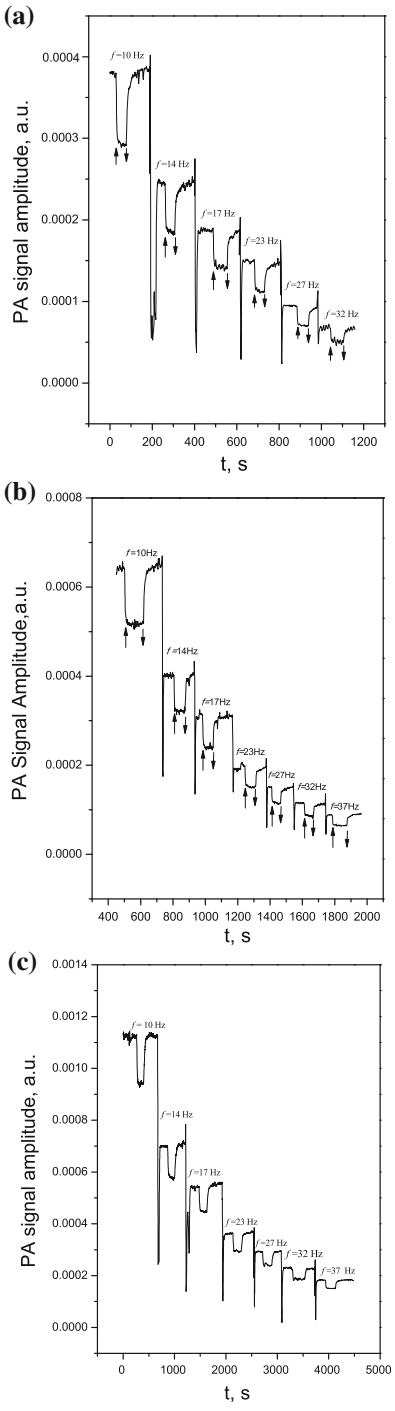

Fig. 2 PA signal amplitude as a function of time (t) for a leaf of maize plant from (a) a control group consisting of, (b) seeds inoculated with *B. unamae* and (c) seeds inoculated with *A. brasilense*. The *arrows* indicate the moment at which oxygen evolution saturating light was turned on (↑) and off (↓)

Table 1 Mean OER values, thermal diffusivity (D_{TH}) and oxygen diffusion coefficient (D_o) for the different groups of treated maize plants

Treatment	D_{TH} ($\mathrm{cm}^2 \cdot \mathrm{s}^{-1}$)	OER (%)	D_o ($\times 10^{-6}\,\mathrm{cm}^2 \cdot \mathrm{s}^{-1}$)
A. brasilense	0.0044 ± 0.0004	21 ± 1	4.2 ± 0.7
B. unamae	0.0062 ± 0.0004	18 ± 1	4.2 ± 0.5
Control	0.0067 ± 0.0003	23 ± 1	5.5 ± 0.6

its value (A) to the one of the pure PT component (A'). Thus, the information obtained from the curve was used for monitoring directly the OER, which was calculated using Eq. (1). The mean values of several independent measurements are listed in Table 1.

Also, the thermal diffusivity and oxygen diffusion coefficient were estimated. In order to determine the thermal diffusivity, we measured the frequency dependence of the PA signal under conditions of oxygen evolution saturation, i.e., we measured the signal S'. Figure 3 shows typical graphs of the logarithm of the $A' \times f$ product as a function of the square root of the modulation frequency. The solid lines depict the best linear least squares fits according to Eq. (3). The value of D_{TH} is calculated from the slope of the graph, namely $d(\pi / D_{\mathrm{TH}})^{1/2}$, using a measured leaf thickness $d = 200\,\mu\mathrm{m}$. Because for the modulation frequencies used here, the measured signal contains the instrumental transfer function due to the frequency-dependent microphone response, this contribution was eliminated using the method described by Ferreira et al. [29] via normalization of the curves of PA signal amplitude vs. frequency using those having a $f^{-1.5}$ dependence, which corresponds to a thermally thin, $12\,\mu\mathrm{m}$ thick, aluminum foil sample.

The same procedure was repeated without saturating the PB component. According to Eq. (2), it was possible to estimate the term $(1/D_0)^{1/2} + (1/D_{TH})^{1/2}$ from the slope of the $\ln(R)$ versus $f^{1/2}$plot (Fig. 4), from which we were able to calculate D_0 using the previously obtained value of D_{TH} and a typical reported literature value of $l = 1\,\mu\mathrm{m}$ [30]. Note that the microphone transfer function was eliminated by the ratio defined in Eq. (2). Table 1 shows the values of both the mass diffusion coefficient and the thermal diffusivity for the three treatments considered in this work.

From Table 1, it is possible to observe that the isolated use of *B. unamae* and *A. brasilense* on maize plants decreases the estimated values of the oxygen diffusion coefficient and of the thermal diffusivity with respect to the control group (without bacteria inoculation). The thermal diffusivity behavior suggests changes in the leaf internal composition due to bacteria inoculation. These changes may be due to the increase in viscosity that causes resistance to oxygen diffusion, as suggested by the reduction in the values of the oxygen evolution rate with respect to those of the control group. The results also show that both treatments cause effects on the photosynthesis process. In particular, we observed the same effect of the two bacteria on the oxygen diffusion coefficient, which becomes approximately 20 % lower than that of the control group plants. The observation that the OER values are different in both cases can probably be an indication of non-photosynthetic factors influencing the measurements, such as the leaf internal anatomy, which is evidenced by the thermal diffusivity behavior.

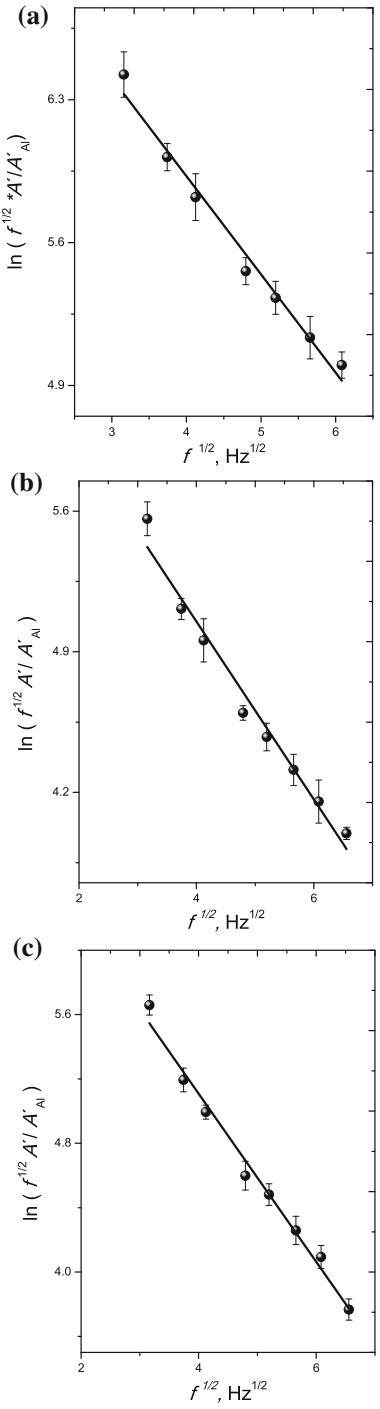

Fig. 3 Frequency dependence of the normalized photoacoustic signal in leaves of maize (a) non-inoculated, (b) inoculated with *B. unamae* and (c) inoculated with *A. brasilense*. The logarithm of the $(f^{1/2} \times A')/A'_{Al}$ is plotted as a function of the square root of the modulation frequency. The term A'_{Al} corresponds to the signal amplitude for a thermally thin sample of aluminum with the characteristics described in text. The *solid line* corresponds to the best least squares linear fit according to the well-known predictions of the RG model

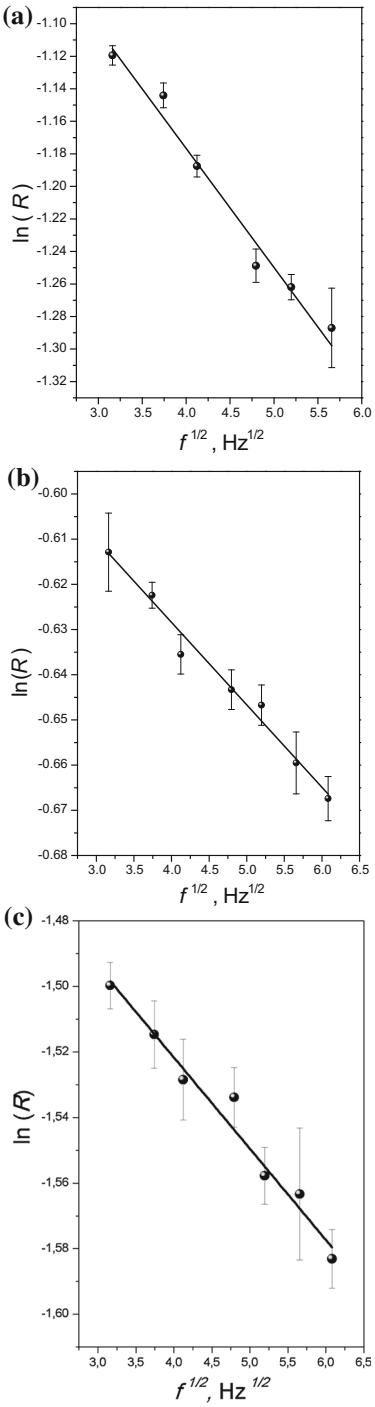

Fig. 4 Frequency dependence of the photoacoustic signal in leaves of maize plants (a) non-inoculated, (b) inoculated with *B. unamae* and (c) inoculated with *A. brasilense*. The logarithm of the amplitude of R is plotted as a function of the square root of the modulation frequency f. The *solid line* corresponds to the best least squares linear fit using Eq. (2)

Although the photoacoustic technique shows to be effective for this kind of research on photosynthesis, via generating and detecting a modulated signal, it is worth mentioning that the modulated oxygen electrode approach [31] is one of the most employed techniques for oxygen evolution measurement. Its sensitivity depends on the electrodes' polarization voltage and on the efficiency of oxygen reduction. As the oxygen concentration can differ between experiments, results obtained with the photoacoustic method and with the modulated oxygen electrode technique are not directly comparable. However, the oxygen diffusion coefficient values measured here are of the same order of magnitude as those reported by other authors in other plant systems [14,30].

4 Conclusions

Photosynthetic activity monitoring by a PA technique was applied on maize plants treated by two different bacterial inoculants. Both treatments are found to have an effect on the photosynthesis process due to plant–microbe interaction. A 20 % reduction in the oxygen diffusion coefficient with respect to the value obtained for the control group plants was found. The experimental conditions guarantee the isolation of other variables considered in conventional field studies. The photoacoustic technique used in this work is a well argued and cheap method that allowed us to perform *in situ* and *in vivo* measurements. The obtained results can contribute to a better understanding of bacterial inoculants effects on plants for biofertilizers development.

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