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Photosynthetic induction in *Eucalyptus urograndis* seedlings and cuttings measured by an open photoacoustic cell

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Abstract

Photosynthetic induction in leaves of four-month-old *Eucalyptus urograndis* seedlings and of cuttings obtained from adult trees that were previously dark-adapted was studied by the *in vivo* and *in situ* Open Photoacoustic Cell Technique. Results for the gas exchange component of the photoacoustic (PA) signal were interpreted considering that the gas uptake component would have a phase angle nearly opposite to that of the oxygen evolution component. By subtracting the thermal component from the total PA signal, we studied the competition between gas uptake and oxygen evolution during the photosynthetic induction. Seedlings presented a net oxygen evolution prior to cuttings, but cuttings reached a higher steady-state photosynthetic activity. The chlorophyll (Chl) *a/b* ratio and the Chl fluorescence induction characteristic F_v/F_m were significantly higher for cuttings, while there was no difference between samples in stomata density and leaf thickness. Thus the differences in PA signals of seedlings and cuttings are associated to differences between the photosystem 2 antenna systems of these samples.

Additional key words: chlorophyll fluorescence; clone differences, gas uptake, leaf thickness; oxygen evolution, photobaric signal; photothermal signal; stomata density.

Introduction

Working with leaf discs and isolated chloroplasts, Inoue *et al.* (1979) simultaneously measured the induction kinetics of the photoacoustic (PA) signal and of the chlorophyll (Chl) fluorescence, showing that the PA transient was associated with photosynthesis. Nowadays, the usefulness of the PA technique in the study of leaf photosynthesis is already established (Fork and Herbert 1993, Malkin and Canaani 1994, Malkin and Puchenkov 1997, Buschmann 1999). The principle of the technique relies on photon absorption and consequent heat and oxygen release. The amplitude (magnitude) and phase (delay with respect to the photon absorption) of the PA signal carry information about optical and thermal parameters of the sample.

The absorbed radiant energy is partially converted into photochemical energy and heat, being partially re-emitted as Chl fluorescence in the photosynthetic process. The PA-signal phase carries information about the delay between photon excitation and pressure variation, which depends on the coefficients of heat and gas diffusion. The signal phase also depends on the time constants of various steps in the electron transfer chain (delayed generation of heat). Poulet *et al.* (1983) determined the oxygen diffusion coefficient and estimated the limiting time constant on the donor side of photosystem 2 (PS2). Korpiun and Osiander (1992) discussed a more complete model of mass diffusion.

Most PA measurements of photosynthesis have been performed with the leaf being cut and enclosed in the cell (Bults *et al.* 1982, Canaani *et al.* 1982, Havaux *et al.* 1987, Malkin 1987, Dau and Hansen 1989, Charland *et al.* 1992). In 1987, an Open Photoacoustic Cell (OPC) was conceived (Perondi and Miranda 1987, Marquezini *et al.* 1991, Pereira *et al.* 1994). A commercial electret

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phone that uses its own chamber as the acoustic cell forms this compact device, with the sample acting as one of the microphone walls. As the leaf itself closes the PA chamber (with the abaxial surface turned to the inside of the chamber), it is not necessary to cut a leaf disc, neither to detach the leaf from the plant for measurements. Thus the OPC allows *in vivo* and *in situ* monitoring of photosynthetic activity in plants, avoiding dehydration of the sample. Part of the leaf remains exposed to the outside, capturing external CO_2, which minimises changes in the PA chamber atmosphere. The OPC technique has been used in studies of, *e.g.*, the effect of dehydration in soybean leaves (Pereira *et al.* 1992), the evidence of heterosis in maize hybrids (da Silva *et al.* 1995), the energy storage determination (Barja and Mansanares 1998), and the effects of irradiance and temperature in *Eucalyptus* leaves (Barja *et al.* 2001).

The two most cultivated species of *Eucalyptus* in Brazil are the fast growing *E. grandis* W. Hill *ex* Maiden and the *E. urophylla* S.T. Blake, that grows less, but is more tolerant to water-limited conditions (Blake 1977, Pryor *et al.* 1995). The crossing of these two species generates the hybrid *E. urograndis*, which presumably grows faster and is more resistant to dry regions (Blake *et al.* 1988, Inoue and Oda 1988). For these reasons, the *E. urograndis* hybrid is nowadays the most planted by reforesting companies in Brazil. Due to the unequivocal economic importance of the *Eucalyptus* culture, it is important to study this hybrid, not completely characterised until now.

In this work, we measured the *in vivo* and *in situ* photosynthetic induction in leaves of *E. urograndis* seedlings and cuttings (the so-called clones), using the PA technique. Our goal was to characterise the photosynthetic behaviour of the *E. urograndis* C041 cuttings, since cuttings are gradually replacing seedlings in reforesting practice due to faster growing in the field and better adaptability to surrounding conditions.

Materials and methods

PA setup: The experimental scheme utilised has two light sources: a Xenon arc lamp (*Oriel*, model *6128*, 1 000 W) and a tungsten lamp (*Ushio/ELC*, 250 W). To obtain modulated radiation of a given wavelength (680±10 nm), we put a chopper (*PAR*, model *192*) and a monochromator (*Oriel*, model *77250*) in front of the Xenon lamp. Measurements were carried out at 17 Hz. Optical filters limited radiation of the tungsten lamp to the visible part of the spectrum. Irradiance by the modulated red radiation used for photosynthetic induction was 10 W m^{-2}, while that of the continuous "white light" used for photosynthesis saturation was about 350 W m^{-2}. A double-branched optical cable guided each radiation beam up to the acoustic cell. The chopper and the PA cell microphone were connected to a lock-in amplifier (*PAR-EGandG*, model *5210*), that selectively amplifies the PA signal taking into account the modulation frequency. The lock-in was connected through a *GPIB* to a microcomputer for data acquisition. Typical time-constant used was 1 s, which gives the time response of the set-up.

OPC is already characterised in the literature (Perondi and Miranda 1987, Marquezini *et al.* 1991, Pereira *et al.* 1992, 1994, Barja and Mansanares 1998). The sensitivity of the electret microphone was about 10 mV Pa^{-1}.

Plants: Both *E. urograndis* seedlings and C041 cuttings (obtained of adult trees) were cultivated in small pots (50 cm^3) under 50 % shade. They were irrigated and fertilised daily. The use of such recipients in the present work follows their current use by the reforesting company that provided the samples. Four-months-old seedlings and cuttings were transferred to the laboratory where they were dark-adapted for at least 10 h at ambient temperature. After this dark period, the plants were moved to the experimental set-up and a selected part of a nondetached leaf was fixed to the OPC and exposed to the radiation coming from the optical cable. Fully expanded leaves of the second pair were selected for measurements. Average values were taken over about 10 measurements for each kind of sample.

Photosynthetic induction: When plants are dark-adapted for a certain time, their photosynthetic reaction centres deactivate. After dark-adaptation, radiation incidence gives rise to the photosynthetic induction, *i.e.*, the restart of photosynthetic processes (Prinsley and Leegood 1986, Malkin 1987, Gardeström 1993, Sassenrath-Cole and Pearcy 1994). Therefore, the initial level of PA signal corresponds solely to temperature oscillation (thermal component). As reaction centres readapt to irradiance, photosynthesis starts and the production of oxygen takes place, giving rise to the so-called photobaric signal (gas component) and eventually reaching the steady state. Simultaneous incidence of modulated (10 W m^{-2}, Xenon lamp) and continuous (350 W m^{-2}, tungsten lamp) radiation gives the maximal thermal component, since the non-modulated radiation saturates the gas exchange component. One must subtract the thermal component from the total PA signal to analyse the photosynthetic activity of the plants studied.

Actually, when only modulated radiation is present, the thermal component is lower than that observed under simultaneous incidence of both modulated and continuous radiation, due to energy storage. However, considering typical energy storage for eucalyptus plants (see Barja and Mansanares 1998) would implicate a correction of about 3 % of the total signal, which does not affect the analysis being made in the present case. For this reason,

we do not present here such correction over the values.

After subtracting the thermal component, the PA signal expresses the photosynthetic gas evolution. Both amplitude and phase of this remaining signal vary with time, reflecting the restart of the photosynthetic processes. The stationary phase value is the oxygen evolution phase angle. As oxygen uptake and evolution are expected to have opposite phase angles, it is possible to study the competition between these processes by looking at the component of the gas signal in the final phase angle. In other words, we project the gas signal amplitude on its final phase (Baesso *et al.* 1989). If the projection is positive, we have net oxygen evolution, on the contrary, we have net gas uptake.

Additional measurements: Chl *a* and *b* contents were determined in the second pair of leaves following the spectrophotometric method of Arnon (1949). Chl *a* fluorescence measurements performed *in vivo* and *in situ* were used to obtain the F_v/F_m ratio. Such measurements were made using a *Hansatech* detector model *PEA* (Plant Efficiency Analyser). Leaf thickness and stomata densities in the abaxial epidermis were also determined: the later one by using a technique of epidermis prints on adhesive.

Results

Fig. 1*A* shows a typical curve of the photobaric signal amplitude in photosynthetic induction measurements in *E. urograndis* seedlings. At time $t = 0$, modulated radiation (10 W m^{-2}, 680 nm, 17 Hz) was switched on and the PA signal (amplitude and phase) was recorded as a function of time. The amplitude of the gas component presented initially a fast increase in the signal amplitude ($t < 20$ s), followed by a decrease and a subsequent increase up to a steady state, reached after about 4 min.

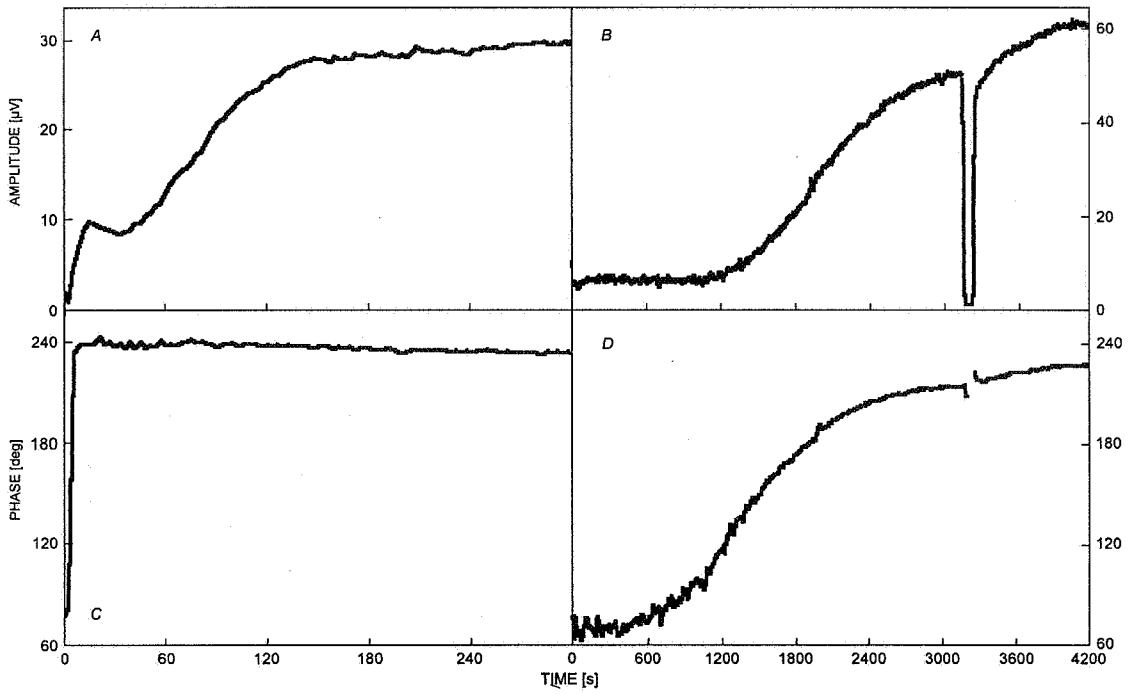

Fig. 1. (*A, B*) Typical photosynthetic induction curves (amplitude of the gas component as a function of time) obtained for *E. urograndis* seedlings previously dark-adapted for $t > 10$ h. *Solid line*: amplitude of the gas component. Modulated radiation characteristics: $\lambda = 680$ nm, modulation frequency of 17 Hz, and irradiance of 10 W m^2. In *B*, continuous irradiation ($400 < \lambda < 700$ nm, 350 W m^{-2}) was switched on at T = 3 200 s (off at $t = 3\,260$ s). (*C, D*) Phase of the gas component in the beginning (up to 300 s after irradiation) of the photosynthetic induction for *E. urograndis* seedlings. Absolute phase values depend on the experimental set-up.

To better understand the beginning of the photosynthetic induction, we must look at the phase of the gas component. Fig. 1*C* shows the phase of this component in the first 30 s under irradiation in *E. urograndis* seedlings: at $t \sim 3$ s, there was a sudden change of almost 180 degrees, with the phase remaining approximately constant after that. This abrupt phase change suggests an initial peak of gas uptake in the photosynthetic induction. Thereafter, the net result of the gas exchange competition between uptake and evolution is an oxygen evolution.

Table 1. Characteristic induction time t_0, initial ($t \leq 30$ s) and steady-state gas component for *E. urograndis* seedlings and cuttings (average over 10 measurements ± standard sample deviation σ_{n-1} divided by $n^{1/2}$).

Parameter	Seedlings	Cuttings
t_0 [s]	90±6	2180±60
Initial gas component [μV]	3.2±0.7	-6.3±0.7
Final gas component [μV]	32±2	67±5

Fig. 1*B* shows the amplitude of the gas component for a typical photosynthetic induction measurement in *E. urograndis* cuttings. The corresponding phase is presented in Fig. 1*D*. The photobaric signal phase in photosynthetic induction of *E. urograndis* cuttings showed a large shift that started at the beginning of the induction and stopped when steady-state was achieved. The occurrence of this phase shift suggests a competition between gas uptake and evolution in which the net result changes from an initial uptake (for $0 \leq t \leq 1\,200$ s) to a subsequent evolution ($t > 1\,200$ s).

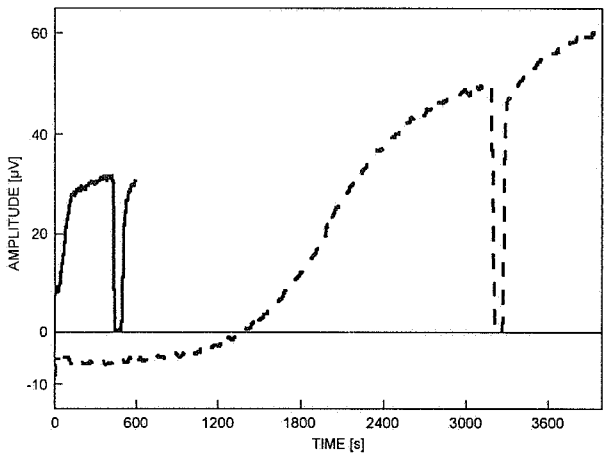

Fig. 2. Projection of the photobaric amplitude on the final photobaric phase value. *Solid line*: *E. urograndis* seedling (same measurement as in Fig. 1*A,C* continuous irradiation of the same characteristics as in Fig. 1*B,D* was switched on at $t = 440$ s, off at $t = 500$ s). *Dashed line*: *E. urograndis* cutting (same measurement as in Fig. 1*B,D*, continuous irradiation was switched on at $t = 3\,200$ s, off at $t = 3\,260$ s).

Table 2. Fluorescence, leaf thickness, stomata density in the abaxial epidermis, chlorophyll (Chl) concentration, and Chl *a/b* ratio for *E. urograndis* seedlings and cuttings (average over 10 measurements ± standard sample deviation σ_{n-1} divided by $n^{1/2}$).

Characteristic	Seedlings	Cuttings (C041)
F_v/F_m	0.804±0.008	0.861±0.004
Thickness [μm]	276±18	303±21
Number of stomata [$\times 10^8$ per m^2]	8.0±1.0	7.6±1.1
Chl *a* [mg m^{-2}]	96±5	110±10
Chl *b* [mg m^{-2}]	57±9	40±10
Chl (*a*+*b*) [mg m^{-2}]	153±14	150±20
Chl *a/b*	1.7±0.2	2.7±0.3

Fig. 2 shows the projections of the photobaric amplitude on the corresponding final photobaric phase for the measurements shown in Fig. 1*A,C* (*E. urograndis* seedling, *solid line*) and Fig. 1*B,D* (*E. urograndis* cuttings, *dashed line*). When the projection was negative, gas uptake was stronger than oxygen evolution. While *E. urograndis* seedlings reached stationary state in 4 or 5 min, photosynthetic induction in cuttings showed net oxygen evolution only for $t > 20$ min, taking about one hour to reach steady-state (Fig. 2).

Regarding the final amplitude of the gas component, Fig. 2 reveals that the steady state photosynthetic activity rate for the *E. urograndis* cutting is about twice that observed for the seedling. We can summarise the differences between seedlings and cuttings as follows: as photosynthetic induction starts, seedlings present a net oxygen evolution earlier than cuttings, but cuttings reach a higher steady-state photosynthetic activity.

Mean values for the characteristic induction time t_0 (time at which the gas evolution reaches 50 % of the steady-state level), the gas component in the initial period of induction ($t \leq 30$ s), and the gas component after achievement of the steady state are summarised in Table 1. Negative value indicates net gas uptake by cuttings.

The amount of Chl (*a*+*b*) was the same in both seedlings and cuttings, but Chl *a/b* was significantly higher for cuttings (Table 2). Stomata density and leaf thickness were similar for both samples, while the ratio F_v/F_m was higher for cuttings (Table 2).

Discussion

The characteristic induction time shows a large difference between seedlings and cuttings. Furthermore, seedlings present net oxygen evolution 3 s after irradiation and forth. On the other hand, cuttings show net gas uptake during the first 20 min. This connects with the delay observed in achieving the steady state. The net gas uptake observed cannot represent CO_2 absorption, since such component is completely damped by the modulation frequency (Bults *et al.* 1982). Reising and Schreiber (1992, 1994) and Reising *et al.* (1998) related PA uptake signals to CO_2 uptake, nevertheless, as was pointed out by Havaux and Malkin (1998), their experiments were performed under "exceptionally high CO_2 levels", which is not the case here. Actually, considering the conditions of our measurements (modulation frequency of 17 Hz, normal atmosphere), we conclude that the gas component of the PA signal reflects (besides oxygen evolution) oxygen absorption processes. Charland *et al.* (1992) do not

discard photorespiration as a possible cause for the O_2 uptake. In this case, similar measurements performed in C4 plants should not present appreciable gas uptake. Indeed, OPC measurements performed in maize plants that show long photosynthetic induction times (da Silva *et al.* 1995) did not uptake gas. However, besides being a slow enzymatic process, the photorespiratory contribution would require some time after the onset of irradiation (since it depends on the activation of ribulose-1,5-bis-phosphate carboxylase/oxygenase and increases with the rise in intercellular O_2 concentration), while Fig. 2 shows a gas uptake component for cuttings since the beginning of the induction. Havaux *et al.* (1987) mentioned the Mehler reaction (oxygen reduction by electrons from PS1) as a source of photoacoustically detected O_2 uptake after heat shock. Working with pulsed photoacoustics, Mauzerall (1990) observed O_2 uptake using far-red radiation (695 nm, absorbed preferentially by the PS1), thus supporting the hypothesis of Havaux. According to Malkin and Puchenkov (1997), negative signals that appear transiently after subjecting the leaf to a long dark period may reflect oxygen photoreduction by PS1, because electron transport starts before activation of the (enzymatic) CO_2 fixation process. In such circumstance, oxygen acts as an electron acceptor to PS1.

Besides the differences in the characteristic induction time and in the O_2 uptake, there is a fast transient in the first minute of the photosynthetic induction clearly seen in seedling but not in cutting measurements. Probably the fast transient in cuttings is being masked by the long-term uptake observed for these samples. The peak observed in the first minute after irradiation has already been reported for leaves of various plants species (Inoue *et al.* 1979, Prinsley and Leegood 1986, Malkin 1987), but has not been fully understood until now. The initial stage of the photosynthetic induction seems to be limited by metabolite contents related to inorganic phosphate (P_i) availability (Sassenrath-Cole and Pearcy 1992, Rao and Terry 1994). Dark-adaptation makes the concentration of 3-phosphoglycerate (PGA) increase as RuBP is consumed. Irradiation induces the reduction of PGA, resynthesised through RuBP carboxylation, CO_2 uptake requires that its acceptor, RuBP, be regenerated. RuBP regeneration depends on ATP, whose synthesis, in turn, depends on P_i availability in the stroma. Our interpretation agrees with the work by Prinsley and Leegood (1986) that relates the transient to a rapid photoreduction of both PGA and P_i stored during the dark period. Furthermore, Sassenrath-Cole and Pearcy (1994) point that the first minute of the photosynthetic induction is governed by RuBP regeneration.

Back to the comparison between seedlings and cuttings, another remarkable difference appears in the steady state signal amplitude: cuttings present signal amplitude about twice that of seedlings (Table 1). The preceding differences were related to induction time and competition between gas uptake and evolution. The analysis of these aspects is unequivocal. Signal amplitude, however, may be affected by leaf morphology. Therefore, it is necessary to investigate in what basis the higher PA signal amplitude in cuttings does reflect a higher photosynthetic activity rate for these samples.

As discussed by Korpiun and Osiander (1992), the amplitude of the gas component in PA signal is *proportional* to (*1*) the effective chloroplasts and cell wall areas involved in the gas exchange process, (*2*) the optical absorption coefficient related to the chlorophyll molar concentration, (*3*) the molar concentration of reaction centres, and (*4*) the quantum yield for the charge separation by an open reaction centre. The signal amplitude is also dependent on the mass diffusion characteristics in the photosynthetic environment. These characteristics are described in terms of the membrane permeability to oxygen, and their effects are pronounced in high modulation frequencies only. Since our measurements were performed at a low modulation frequency (17 Hz), the influence of possible differences in morphology between seedlings and cuttings, specifically membrane thickness and permeability, is negligible.

Analysing the morphological parameters of Table 2, specifically thickness and density of stomata, we conclude that they are not responsible for the differentiation between seedlings and C041 cuttings. If strong differences in these parameters were observed, they would affect the relative amplitudes of the PA signal through the effectiveness of the coupling between internal pressure variation (in the intercellular volume) and the cell chamber pressure (detected by the microphone). This is not the case. Now, let us analyse the effective areas of each kind of sample referred in (*1*). In order to do this, we cite the results obtained by Blake *et al.* (1988) for *E. grandis* plants about 15 months-old using the infrared gas analyser (IRGA) technique. They show that cuttings present higher photosynthetic activity rate than seedlings. Since the measurements in this case were performed under continuous irradiation, the gas concentration thus determined accounts for the total gas production. This quantity is independent of the effective chloroplasts and cell wall areas, since all the gas produced and consumed crossed the membranes. Hence, by assuming correspondence between IRGA measurements (for *E. grandis*) and PA measurements (for *E. urograndis*), one must reject membrane area as an element of PA signal distinction.

There are, however, two significant differences between seedlings and cuttings shown in Table 2: F_v/F_m and the Chl *a/b* were both higher for cuttings. The fluorescence parameter gives the maximum quantum yield of the electron transport system, values of about 0.8 are considered normal. The Chl *a/b* ratio, lower for seedlings, indicates that these samples present a PS2 antenna system more developed than in cuttings. At this point, one must consider that, as the cuttings were generated from adult trees, their behaviour may be inherently different from that of seedlings. In principle, being adapted to high (open field) irradiance, adult trees tend to present an antenna system less developed than in low-irradiance-

grown seedlings: this can explain the difference between cuttings and seedlings in the Chl *a/b* ratio.

The difference between the induction kinetics of seedlings and cuttings may reflect a difference between the compensation irradiance of these samples. Shade plants have lower compensation irradiance and lower maximal photosynthetic rates than sun plants (Björkman 1981). As our cuttings were generated from adult (sun adapted) trees, they probably required higher irradiance to promptly start up the photosynthetic processes.

For the given growing conditions, the PA technique was able to distinguish between the two kinds of samples, which presented specific patterns of photosynthetic induction. Hence we conclude that the differences observed in the signal amplitude in steady state do represent a higher photosynthetic activity rate for cuttings, related to pigment (antennae) concentration and to the photochemical quantum yield. In other words, the higher PA signal does represent a higher photosynthetic activity, and the OPC technique is a valuable tool to establish distinctions in the photosynthetic performance of different plants. In principle, the higher photosynthetic rate presented by cuttings is more relevant for the development of the plants than the faster induction shown by seedlings (Edwards and Walker 1983).

Our results for cuttings correspond exclusively to the C041 line studied. However, PA measurements performed in C219 and C031 cuttings (values not shown) indicate that the higher photosynthetic activity is a general behaviour of the *E. urograndis* clones. For all these cuttings, the amplitude of the steady-state PA signal was clearly higher than that of the seedlings.

Our study showed the usefulness of the OPC in investigating the photosynthetic behaviour of *E. urograndis* leaves. While seedlings presented net oxygen evolution a few seconds after the onset of irradiation, such a net result took at least 20 min to be observed for C041 cuttings. While seedlings presented a faster induction, cuttings reached a much higher steady-state photosynthetic activity rate. This result is important in the planning of ecologically and economically advantageous *Eucalyptus* agriculture. The fact that *E. urograndis* cuttings show a higher photosynthetic activity rate than *E. urograndis* seedlings of the same age has not been shown yet using the PA technique. The present work opens various perspectives of study within this field, such as a long-term analysis of the open-field performance of *E. urograndis* cuttings and seedlings.

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