View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Repositorio da Producao Cientifica e Intelectual da Unicamp

Photoacoustic Spectroscopy for Depth-profile Analysis and Herbicide Monitoring in Leaves

José W. Nery, Osvaldo Pessoa, Jr., and Helion Vargas
Instituto de Física, Universidade Estadual de Campinas, 13100 Campinas, SP, Brazil
Francisco de A. M. Reis
Instituto de Química, Universidade Estadual de Campinas, 13100 Campinas, SP, Brazil
Antonio C. Gabrielli
Instituto de Biologia, Universidade Estadual de Campinas, 13100 Campinas, SP, Brazil
Luis C. M. Miranda
Laboratório Associado de Sensores e Materiais, Instituto de Pesquisas Espaciais, 12200 São José dos Campos, SP, Brazil
Carlos A. Vinha
Departamento de Física, Universidade Federal do Mato Grosso do Sul, 79100 Campo Grande, MS, Brazil

Published on 01 January 1987. Downloaded by UNIVERSIDAD ESTADUAL DE CAMPINAS on 25/06/2015 14:48:07.

Depth profiles of double-layer biological samples obtained by photoacoustic spectroscopy were studied using the two-signal phase-resolved method. The application of the method was demonstrated by singling out the spectra of the cuticle and the pigment layers of a leaf, and the pericarps and the endosperm layers of a corn kernel. The use of the method for monitoring temporal changes occurring in a leaf under the action of a herbicide was also investigated.

Keywords: *Phase-resolved photoacoustic spectroscopy; corn leaf spectra; herbicide action; depth-profile analysis*

In the last decade, photoacoustic spectroscopy (PAS) has attracted the attention of researchers from fields ranging from materials science to biology. Photoacoustic spectroscopy is based on the production of an acoustic wave in a closed cell containing air in contact with a sample exposed to monochromatic chopped light. The source of the acoustic signal is the periodic heat flow from the sample to the surrounding gas as the sample is cyclically heated by the absorption of the chopped light. The periodic flow of this heat into the gas cell produces pressure fluctuations which are detected as an acoustic signal. The wavelength of the incident chopped light can be varied, and the detected acoustic signal is analogous to that from conventional spectroscopic techniques. A more detailed account of PAS and its applications has been given by Rosencwaig.[1]

Photoacoustic spectroscopy has the unique capability of resolving the spectra of each constituent in a multi-component or layered system. This depth-profiling capability arises from the fact that the PA signal is sensitive only to the heat generated within one thermal diffusion length [$\mu = (\alpha/\pi f)^{\frac{1}{2}}$] beneath the surface. In this expression, α is the thermal diffusivity of the medium and f is the modulation frequency of the heating light beam. Hence by varying the modulation frequency (*i.e.*, varying the thermal diffusion length), depth-profile analyses can be performed. This conventional technique has been used for studying layered samples such as layered tape,[2] colour photographic film,[3] leaf samples[4,5] and the distribution of pigments in lobster shells.[6] This technique has, however, some experimental difficulties, owing to a poor signal to noise ratio. This difficulty is overcome by using the recently proposed two-signal phase-resolved method (PRM) as an alternative technique for depth-profile analysis. This method was independently developed for biological[7,8] and solid[9,10] samples.

In this paper we explore the use of the phase-resolved method described by Cesar and co-workers[9,10] for depth-profile analysis and monitoring of the action of herbicides in plants. As is well known, different chemicals interfere in various stages of the photosynthesis process and the study of their mechanism of action requires some elaborate analytical procedures; PAS offers a simple alternative method for assessing the action of herbicides in leaves.

Theory

The basics of PRM may be summarised as follows. Let us consider a typical PA arrangement in which heat is generated within the sample owing to the absorption of chopped radiation. We further assume that the sample is made of two layers of materials A and B, with material A facing the gas in the PA cell. At a fixed modulation frequency, the acoustic signal detected at the microphone is a result of the contributions generated in both constituents A and B. As component B is beneath A there should be a delay between the signals arising from A and B due to the difference in the corresponding thermal diffusion times. This difference in the time taken to reach the gas produces a phase shift, ψ, between the two signals. Hence, the signal S actually observed may be viewed as the resultant of two vectors (whose lengths S_A and S_B correspond to the signals from A and B, respectively) with an angle ψ between them. This means that once the angle ψ is known, by, say, varying the phase angle by 90° with respect to S_A, only the contribution of component B, and *vice versa,* will be observed. In other words, by measuring the phase variation of the PA signal of a composite sample it is possible, in principle, to single out the contribution of the various constituents at different locations. The different sub-surface layers are then identified in the PRM by the transit time of the heat generated at a single modulation frequency. The spectrum may be phase-resolved as follows. The in-phase (S_o) and quadrature (S_{90}) signals of the sample are recorded as a function of the incident light wavelength. The spectrum at a given phase ϕ is written in terms of $S_0(\lambda)$ and $S_{90}(\lambda)$ as

$$S_\phi(\lambda) = S_o(\lambda)\cos\phi + S_{90}(\lambda)\sin\phi \quad .. \quad .. \quad (1)$$

View Article Online

Published on 01 January 1987. Downloaded by UNIVERSIDAD ESTADUAL DE CAMPINAS on 25/06/2015 14:48:07.

Experimental

Photoacoustic Equipment

The following equipment was used in our laboratory-made PA spectrometer: a 1000 W xenon arc lamp from Oriel, a BK-4166 condenser microphone from Bruel and Kjaer, a monochromator from Jarrel-Ash, a rotating blade light chopper from PAR, lock-in amplifiers from PAR, a Hewlett-Packard register and a Commodore microcomputer. The PA cell used was a laboratory-made brass cell.

Materials

Coffea arabica, Glycine max and *Datura stramonium* plants were kindly provided by Dr. J. G. Cortes from the Instituto Brasileiro do Café (IBC) at Campinas. The herbicide used was a commercial formulation of paraquat (Gramoxone, ICI Chemicals). Sources of other materials and equipment are as indicated in the text.

Results

Depth-profile Analysis

The first experiment conducted was a depth-profile analysis of a green leaf. The sample used was an intact coffee leaf cut in the form of discs of 5 mm diameter. After recording the two spectra at quadrature, S_o and S_{90}, at a fixed modulation frequency of 25 Hz, the signal at a given phase ϕ was calculated using equation (1). Fig. 1(*a*) shows the in-phase spectrum of the leaf with the characteristic absorption bands of the waxy cuticle, caroteneoids and chlorophyll. From Figs. 1(*c*) and 1(*d*) it can be seen that the spectra corresponding to the pigment layers and the cuticle could be isolated at the $\phi = -30°$ and $\phi = 80°$ phases, respectively. This demonstrates the ability of PRM to perform depth-profile analysis of layered structures. To explore further the potential of the PRM we applied this method to the analysis of a corn kernel with a reddish envelope. The spectra were taken at 20 Hz in the range 300–700 nm, and are shown in Figs. 2(*a*) and (*b*), in which the pericarp and endosperm spectra are best resolved at $\phi = -30°$ and $\phi = 70°$, respectively, giving a phase shift of 80°. To confirm that the pericarp and endosperm spectra had been singled out, the phase-resolved spectra were correlated with those of the mechanically isolated layers [Figs. 2(*c*) and (*d*)] as described by Cesar and co-workers.[9,10]

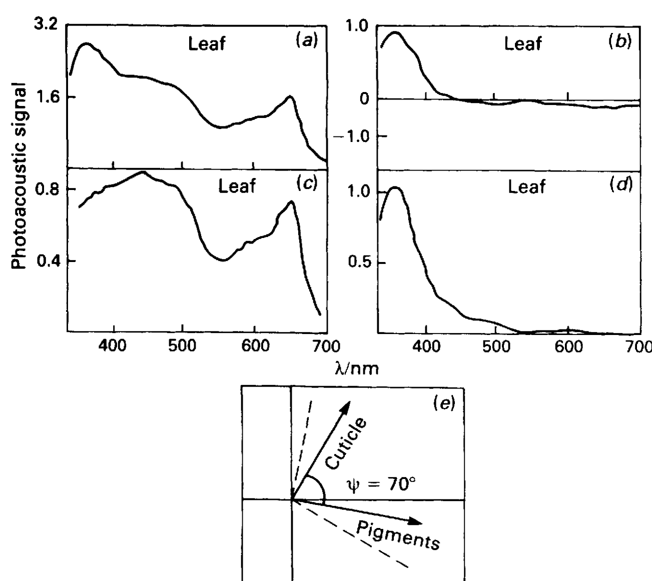

Fig. 1. PRM applied to a coffee leaf. (*a*) In-phase signal S_o; (*b*) quadrature signal S_{90}; (*c*) pigments spectrum resolved at $\phi = -30°$; (*d*) cuticle spectrum resolved at $\phi = 80°$; and (*e*) cuticle signal at phase $\phi_A = 60°$ and pigment signal at phase $\phi_B = -10°$, resulting in a phase difference of 70°

Monitoring Herbicide Action in Leaves

We next applied the PRM in the investigation of the mechanism of action of paraquat in green leaves. Paraquat is a well known contact herbicide acting on photosystem I, inhibiting the electron flow and consequently the phosphorylation process.[11] The plants tested were sprayed with a 5% aqueous solution of paraquat under normal sunny field conditions. As in the previous instance, the samples taken from the plants were cut in the form of discs of 5 mm diameter. Using the same procedure as above, we have resolved the PA spectra of our samples and measured the phase difference $\psi = \phi_A - \phi_B$ between the cuticle and pigment layers, just before the application of paraquat and at 2, 4 and 6 h afterwards. After each measurement the samples were checked for any structural changes using an optical microscope (60× magnification). Figs. 3(*b*) and (*c*) show the phase-resolved spectra of *Glycine max* (soybean) as a function of time elapsed after the paraquat application. Besides the apparent decrease in the amount of pigments with time, the phase shift also exhibited a progressive decrease. Starting from 50°, just before the action of paraquat, ψ decreased to 40°, 30° and 20° after 2, 4 and 6 h of spraying the herbicide, respectively. Two other trials (one with coffee and the other with datura) showed similar behaviour. The observed decrease of the phase shift ψ can be explained by a hypothesis of continuous dehydration of the leaf as follows. The phase shift ϕ_A in the cuticle is given by $a_s x_A$ where a_s is the thermal diffusion coefficient and x_A is the mean cuticle thickness. Assuming that both the cuticle and the pigment layers have the same thermal diffusivity (approximately that of water) and denoting by x_B the mean thickness of the pigment layer, the phase difference can be written as

$$\psi = a_s(x_B - x_A) \quad (2)$$

where $a_s = (\pi f/\alpha)^{\frac{1}{2}}$.

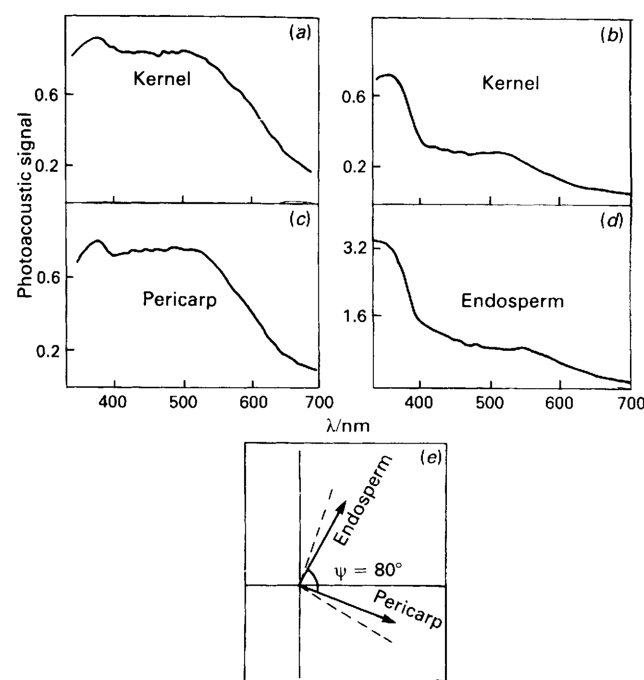

Fig. 2. PRM applied to a corn kernel having a reddish envelope. (*a*) Pericarp spectrum resolved at $\phi = -30°$; (*b*) endosperm spectrum resolved at $\phi = 70°$; (*c*) spectrum of a mechanically isolated pericarp; (*d*) spectrum of a mechanically isolated endosperm; and (*e*) phase difference found, $\psi = 80°$

View Article Online

Published on 01 January 1987. Downloaded by UNIVERSIDAD ESTADUAL DE CAMPINAS on 25/06/2015 14:48:07.

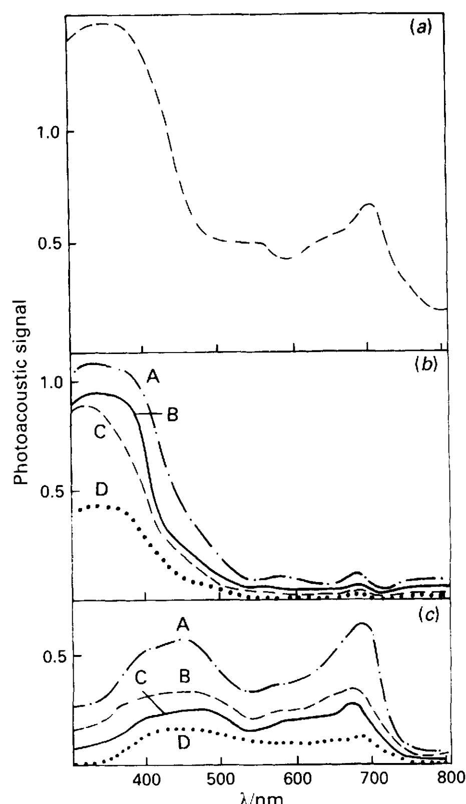

Fig. 3. PRM applied to soybean leaf. (*a*) In-phase spectrum before treatment; (*b*) cuticle and (*c*) pigments spectrum (A) before, (B) 2, (C) 4 and (D) 6 h after treatment

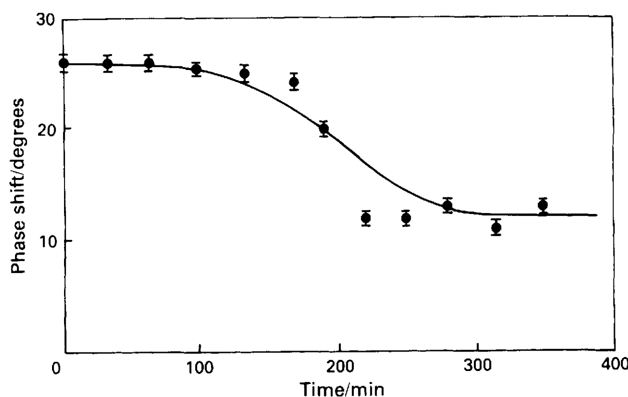

Fig. 4. Phase-shift between the cuticle and the pigment layers in *Glycine max* as a function of time

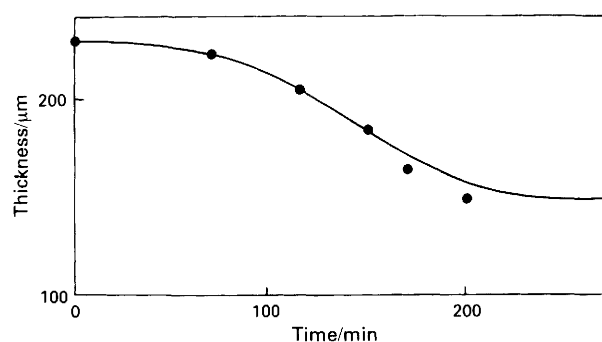

Fig. 5. *Glycine max* leaf thickness change as a function of the time elapsed after the action of paraquat

To check this hypothesis we carried out the phase shift measurements in a more closely spaced time interval on another freshly sprayed soybean leaf sample. The results for the phase shift ψ between the cuticle and pigment layers are shown in Fig. 4. This data, together with equation (2) for the phase shift, suggests that the dehydration induces leaf shrinkage. In fact, in a carefully carried out study[12] on the desiccation-induced alterations in sunflower leaves, water loss resulted in the shrinkage of cells, vacuoles and intercellular spaces causing a decrease in leaf thickness. This decrease in leaf thickness would be expected for any mesophytic plant such as soybean. This was checked for the soybean leaf example by carrying out an *in vivo* monitoring of the soybean leaf thickness under the action of paraquat. The measurements were obtained using an optical microscope with the leaf kept between two glass plates and artificially illuminated with a 160 W tungsten filament lamp. The change in leaf thickness as a function of the time elapsed after the application of paraquat is shown in Fig. 5. The close resemblance between the shapes of Figs. 4 and 5 suggests that the action of paraquat on the soybean leaf is to cause a shrinkage of the leaf owing to the induced dehydration, which is reflected in a decrease of the phase shift ψ as shown in Fig. 4. Parallel to the leaf's dehydration, as evidenced by the decrease of the phase shift ψ, paraquat has a toxic effect owing to the generation of hydrogen peroxide by the redox process induced by the electron transfer from photosystem I. The generation of H_2O_2 is responsible for the destruction of pigments. This destructive role of paraquat is evidenced by the gradual decrease of the intensity of the pigment bands.

Conclusions

In this paper we have described the use of the two-signal phase-resolved method for carrying out time evolution depth-profile analysis of layered biological samples. The method essentially involves the simultaneous recording of the in-phase and quadrature PA signals at a single modulation frequency. With the experimental values obtained, the contributions of the various components can be singled out with the aid of a computer by the variation in phase angle of the resultant signal as given by equation (1). The method has been experimentally tested by resolving the spectra of leaves and corn kernels and by monitoring the action of a herbicide (paraquat) in the degradation of a green leaf. It was shown that the action of paraquat on the leaf is two-fold. Paraquat induces a rapid dehydration in the leaf, as evidenced by the rapid decrease of the PA phase shift between the cuticle and the pigment layers. Parallel to this dehydration process, paraquat increases the phototoxicity in the leaf through the production of hydrogen peroxide, as observed by the gradual decrease in intensity of the pigment absorption bands.

In conclusion, this simple and straightforward method may become a useful technique for plant physiologists as it is used in other investigations of the interaction of chemicals with biological systems.

We thank Prof. W. J. da Silva from UNICAMP for providing us with the corn kernel samples used in this work and to the IBC (Instituto Brasileiro do Café) for financial support. Two of us (O. Pessoa, Jr., and J. W. Neri) are grateful to the CNPq (Brazilian National Research Council) for a scholarship.

References

1. Rosencwaig, A., "Photoacoustics and Photoacoustic Spectroscopy," Wiley, New York, 1980.

View Article Online

Published on 01 January 1987. Downloaded by UNIVERSIDAD ESTADUAL DE CAMPINAS on 25/06/2015 14:48:07.

2. Betteridge, D., Liller, T. C., and Meyler, P. J., *Fresenius Z. Anal. Chem.*, 1979, **296**, 28.
3. Helander, P., Lundström, I., and McQueen, D., *J. Appl. Phys.*, 1981, **52**, 1146.
4. Kato, K., Ishino, S., and Sugitani, Y., *Chem. Lett.*, 1980, **20**, 783.
5. Adams, M. J., and Kirkbright, G. F., *Analyst*, 1976, **102**, 281.
6. Mckenthum, M. L., Tom, R. D., and Moore, T. A., *Nature, London*, 1979, **279**, 215.
7. Anjo, D. M., and Moore, T. A., *Photochem. Photobiol.*, 1984, **39**, 635.
8. O'Hara, E. P., Tom, R. D., and Moore, T. A., *Photochem. Photobiol.*, 1983, **38**, 709.
9. Cesar, C. L., Vargas, H., Pelzl, J., and Miranda, L. C. M., *J. Appl. Phys.*, 1984, **55**, 3460.
10. Cesar, C. L., Vargas, H., and Miranda, L. C. M., *J. Phys. D*, 1985, **18**, 599.
11. Moreland, D. E., *Annu. Rev. Plant Physiol.*, 1980, **31**, 597.
12. Fellows, R. J., and Boyer, J. S., *Protoplasma*, 1978, **93**, 381.

Paper A7/63
Received February 23rd, 1987
Accepted May 21st, 1987