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Photoacoustic Study of Fungal Disease of Acai (*Euterpe oleracea*) Seeds

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Abstract Photoacoustic spectroscopy is introduced as a promising experimental technique to investigate fungus infected Acai (*Euterpe oleracea*) seeds. Photoacoustic spectra of healthy and infected Acai seeds with the fungus *Colletotrichum gloeosporioides* were recorded firstly in the modulation frequency range of 5 Hz to 700 Hz, while keeping the wavelength of excitation radiation of a Xe arc-lamp constant, to ascertain the depth of penetration of infection within the seed and secondly, at variable wavelength (wavelength scanning) in the interval 250 nm to 1,000 nm, while keeping the modulation frequency constant. In the former, the photoacoustic signal strength from the infected seed was found higher than that of the healthy one, and has been associated with the appearance of new biomolecules associated with the pathogen infection. In the latter, characteristics peaks and bands were observed in the range from 650 nm to 900 nm ascribed to organic compounds with carboxylates and amines (functional groups) forming the typical metabolic structures of the fungus.

Keywords Acai (*Euterpe oleracea*) seeds · Fungi · Photoacoustics

1 Introduction

Photoacoustic spectroscopy (PAS) is one of competing modern methods. Principle of PAS was discovered by Bell [1]. The fraction of incident chopped radiation, when

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absorbed by the sample, raises the molecules of the sample from the electronic ground state to the excited electronic state, and these excited molecules relax to the ground state through non-radiative de-excitation, i.e., periodic heat emission. This periodic heat emission produces varying pressure, which can be detected by a microphone as a photoacoustic signal. Rosenwaig and Gersho [2] developed the theoretical explanation of PAS. No special sample preparation is required to obtain photoacoustic spectra, and the photoacoustic signal allows sample characteristics differentiation at different depths, i.e., depth profiling. PAS has been employed in the study of several problems in agriculture, e.g., fungal disease of wheat and rice [3,4], mycorrhizal infection on the photosynthesis of corn [5], photorespiration and temperature dependence of oxygen evolution in tomato plants [6], detection of nitric oxide in tobacco [7], discrimination of transgenic and conventional soybean seeds [8], and so on. Recently, PAS has also demonstrated its value for the studies of Baru (*Dipteryx alata* Vog) fruits from Brazilian Cerrado [9] and surface-coating layers of magnetic nanoparticles [10].

This article is a report of our study of Acai (*Euterpe oleracea*) palm fruits from the Amazon forest using PAS. Acai seeds (they have high potential as biofuel) are of great interest for their use in handcrafts (collar, necklaces, ear rings, to name a few). However, these handcrafts can pose a serious health hazard to humans if the seeds are fungus infected, during the manufacturing process that may cause allergic rhinitis, dermatitis, mycotic keratitis, mycotic infection in the lungs and kidneys subcutaneous hyalohyphomycosis, and so on. Sorting systems based on optical-acoustic methods have the potential to rapidly detect and physically remove seeds severely contaminated by fungi, or infested internally by insect larvae [11]. PAS could be of great help in monitoring the degree of pathogen infection by performing a depth profiling analysis of the infected seeds.

Accordingly, the photoacoustic spectra of Acai seeds infected with the fungus *Colletotrichum gloeosporioides* and those of healthy seeds were measured and compared to obtain information relative to the degree of fungus infection within the seeds. By Acai healthy seeds, we mean seeds treated with specific natural oils. When fungus infected, the pathogen degrades the seed components by means of enzymes, releasing chemical compounds.

2 Material and Methods

2.1 Apparatus

The photoacoustic spectrometer is composed of a 150 W xenon-arc lamp (Oriel Corp., Stratford, CT) with a scanning monochromator (Spex Model 1680). The monochromatic light was modulated by a variable rotating chopper (Stanford SR 540) and was incident on a mirror and passed through a transparent window to reach the sample contained in the cell (see Fig. 1). The PA signal coming from a highly sensitive ($50\,\mathrm{mV}\cdot\mathrm{Pa}^{-1}$) microphone (Brüel & Kjær, Type 4189) as an electrical signal was amplified by a lock-in amplifier (Stanford SRS-Model SR 830), and the synchronized signal was recorded. The microphone has a flat response (0 dB) in the frequency interval from 10 Hz to 10000 Hz (our working frequency range is 5 Hz to 700 Hz), thus

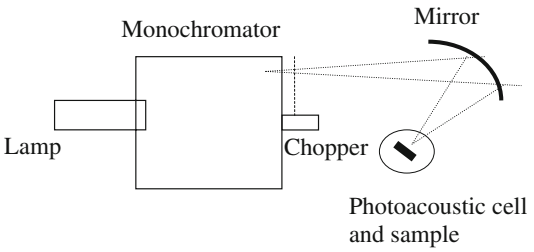

Fig. 1 Photoacoustic experimental setup and cell

giving a linear response to the signal. This microphone has been used in a transducer electronic data sheet (TEDS) combination with a classical 1.27 cm (0.5 in) preamplifier (Type 2669). The TEDS is programmed with the loaded sensitivity of the actual cartridge, and the data can be readily recorded.

2.2 Standardization

Because the light source does not supply constant energy with long-term use or at various wavelengths, the crude spectrum is standardized by computing the ratio of the crude spectrum of a sample to that of carbon black. Carbon black is used because it absorbs light at all wavelengths, and its spectrum represents the profile of the light energy for the apparatus. This manipulation results in standardized absorption spectra related to the incident energy of the light source.

2.3 Material Preparation

Figure 2 shows Petri dishes containing laboratory *C. gloeosporioides* fungus-infected Acai seeds (top) and healthy (treated) seeds. In order to insure that only the *C. gloeosporioides* fungi are infecting the seeds and no additional fungal species with considerable quantity of pigments are present, laborious contamination control was performed. The Acai seed samples are naturally colonized by several fungal species that cause post-harvest damage. However, most of these fungi are associated with the seeds, but they do not infect their interior, colonizing only their surface. Through optical microscopy, these fungi were identified inclusively as the *Colletotrichum*. After the detection of this pathogen which is transmitted by the seeds, we proceeded using the following methodology. The Acai seeds were first washed in ordinary water with soap and then with distilled water to remove the superficial fungi. Next, the seeds were de-infected with sodium hypochlorite for 2 min and washed with distilled and sterilized water thrice and dried on sterilized filter paper (a semi-permeable paper). The seeds were then placed inside moisture chambers (small plastic boxes) with filter papers on the bottom (germ-test) moistened with distilled, sterilized water. The seeds (ten seeds per box) were placed equidistant from each other over the filter paper to avoid eventual contamination and were incubated for 10 days at temperatures of 25 °C to 28 °C. After the seventh day, we observed, under stereoscopic microscopy, the fungus mycelium, and on the tenth day, the *Colletotrichum* acervula with conidia, typical of genre, was

Fig. 2 Petri dishes containing *C. gloeosporioides* fungus-infected Acai seeds (*top*) and healthy seeds (*bottom dishes*)

detected. Through optical microscopy, the *C. gloeosporioides* has been fully identified. This pathogen is seed systemic in that it colonizes internally, and produces fructification on seed surfaces causing progressive deterioration [12]. Since there is no healthy Acai seed in nature, because they are collected from the ground as waste (after the pulp has been extracted), we produced the healthy samples after the above treatment by brushing them with a natural oil solution [13] (comprised basically of Eucalyptus oil, Copaiba oil, Andiroba oil, and Canadian balsam). Independent of whether the treated seeds are pigmented or not, which depends upon the maturity of them, they were put in Petri dishes containing *C. gloeosporioides* fungus colonies and we observed that they were not colonized by the pathogen.

From the top Petri dish, we selected an infected seed and cut it carefully with dimensions of 6 mm in diameter and 1 mm in thickness. The sample was then enclosed in a sealed, high-performance PA cell chamber (cell) containing a gas (air) that was maintained at atmospheric pressure and room temperature (300 K), and was coupled to a highly sensitive microphone.

3 Measurements

Two different approaches have been used for recording the photoacoustic spectra of fungus contaminated Acai seeds. Firstly, the modulation frequency was varied and the wavelength of radiation was kept constant. This technique is called depth profile analysis. The depth of the thermally active layer of the sample is given by the expression [2],

$$\mu = (2k/\omega\rho \mathrm{C})^{1/2}, \tag{1}$$

where μ is the sample thermal diffusion length, k is the thermal conductivity of the Acai seed, ρ is the seed density, C is the specific heat of the seed, and ω is the chopper frequency. The length of the thermally active layer L is given by $L = 2\pi\mu$. The thermally active layer, L, is increased by decreasing the modulation frequency within the sample. This means that for an Acai seed at a lower modulation frequency, the

contribution to the photoacoustic signal comes from the infected inner layers, whereas at a higher frequency, the contribution to the photoacoustic signal comes from the infected outer layers.

In the second approach, the photoacoustic spectra can be recorded by varying the wavelength of the exciting radiation while keeping the modulation frequency constant. This is called wavelength scanning and is expected to give characteristics peaks and bands [9,10].

In order to get the infection depth profile, the photoacoustic spectra of Acai seed samples were recorded by varying the chopping frequency from 10 Hz to 700 Hz and the frequency of radiation was kept at 420 nm. The signal was amplified by a preamplifier and then processed by the lock-in amplifier whose sensitivity was 20 mV, and the time constant was 1 s for all measurements. The measuring time was 5 min.

The fixed wavelength (420 nm) of excitation radiation from the monochromator with 0.02 nm resolution, especially at 420 nm, passed through the chopper and went into the cell. The modulated radiation was then absorbed by the infected seed sample and generated heat and pressure variations inside the cell. The pressure variations created a pressure wave, which was detected by the sensitive microphone. The microphone signal (photoacoustic signal), directly proportional to the light power (or optical absorption coefficient) and inversely proportional to the square root of the chopper frequency and gas volume in the cell, was post-processed and plotted as a function of the chopper frequency. The same procedure was carried out for the healthy sample.

4 Results and Discussion

Photoacoustic spectra are reported as the ratio of two electric signals generated by the microphone, i.e., the crude spectrum of the sample divided by that of carbon black. Thus, the photoacoustic intensity is expressed in relative units. Unfortunately, it is impossible to determine absolute values using the PAS method [14]; it only shows relative changes [15]. This is because the absolute photoacoustic signal is a function of, among other factors, the physical form of the sample; thus, Acai seed samples were used as small pastilles 6 mm in diameter and 1 mm in thickness to standardize its form.

In the first approach (depth profile), the PA spectra of infected and healthy seeds are shown in Figs. 3 and 4. To ascertain the depth of penetration of infection within the seed, we use Eq. 1. To our knowledge the thermophysical properties of the Acai seed kernel are nonexistent in the literature. However, a thermal study of Acai pulp [16] indicated that the measured thermal conductivity (k), specific heat (C), density (ρ), and thermal diffusivity (a) are $k = 0.575\,\mathrm{W\cdot m^{-1}\cdot K^{-1}}$, $C = 3.69\,\mathrm{J\cdot g^{-1}\cdot K^{-1}}$, $\rho = 1.032\,\mathrm{g\cdot cm^{-3}}$, and $a = 1.51 \times 10^{-7}\,\mathrm{m^2\cdot s^{-1}}$. Inserting these values into Eq. 1, the result indicates the depth at 10 Hz to be 1086.3 μm (see Table 1). Since the Acai fruit contains pulp, fibers, and the seed kernel itself, we tend to believe that our depth of our study is of the same order. Yet, the fatty acid composition, determined by gas chromatography revealed the predominance of oleic acid in Acai pulp as much as in the seed kernel (56.2 % of total fat) [17,18]. The measured thermal properties of oleic acid at 25 °C indicate [19] $k = 0.17\,\mathrm{W\cdot m^{-1}\cdot K^{-1}}$, $C = 0.57\,\mathrm{cal\cdot g^{-1}\cdot K^{-1}}$, and $\rho = 0.8935\,\mathrm{g\cdot cm^{-3}}$. Inserting these values into Eq. 1, the result indicates the depth at 10 Hz to be 794.3 μm (see

Table 2). It is clear from Table 1 that for a modulation frequency of 10 Hz, the signal is coming from the overall thickness (about 1 mm). If one uses the data of Table 2, the signal at 10 Hz is coming from part of the sample thickness (about 0.8 mm). This means that the depth of penetration of the infection within the seed at 10 Hz from the upper surface varies from 0.8 mm to 1 mm, depending on the thermal properties of the seed compounds (oleic acids and polysaccharides). It is clear from Figs. 3 and 4 that the PA signal strength was higher in a diseased seed in the frequency range of 10 Hz to 100 Hz and above. The strength of the PA signal coming from the infected Acai seed layer at 383.7 μm (280.7 μm) from Table 1 (Table 2) from the upper surface of the Acai seed is about a factor of six higher than that of the PA signal of a healthy seed at the same depth. The difference in the PA signals of D3 (fungus infected) and D2 (treated with natural oils) samples is due to typical material biomolecules coating the seeds. It suggests that some new biomolecules (probably amino acids present in the fungus spores) were formed in the external and internal layers of the seed associated with pathogens. These new biomolecules synthesized in the course of the disease development absorb extra photons whose energy raises the molecules to an excited electronic state. These excited molecules then relax to the ground state through non-radiative de-excitation (heat emission), thereby enhancing the PA signal. A similar effect was reported for corn-infected seeds with fungus *Fusarium moniliforme* [20].

It is important to point out here that the infection progress deep in the Acai seed certainly depends on the infection time as the fungus goes further into the seed core. It would be interesting to investigate the PA signal versus the modulation frequency ω at different infection times.

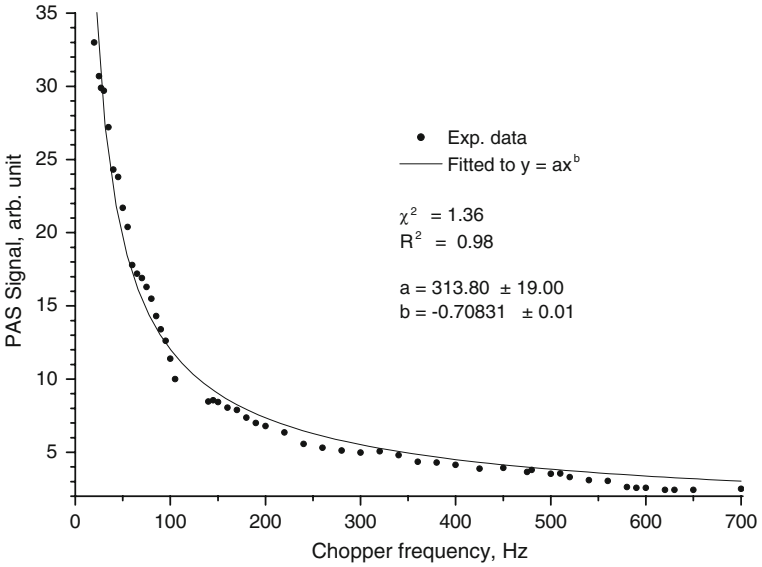

Fig. 3 Photoacoustic signal (in relative units) of infected Acai seed (D3). *Solid line* represents a least-squares fit to the data

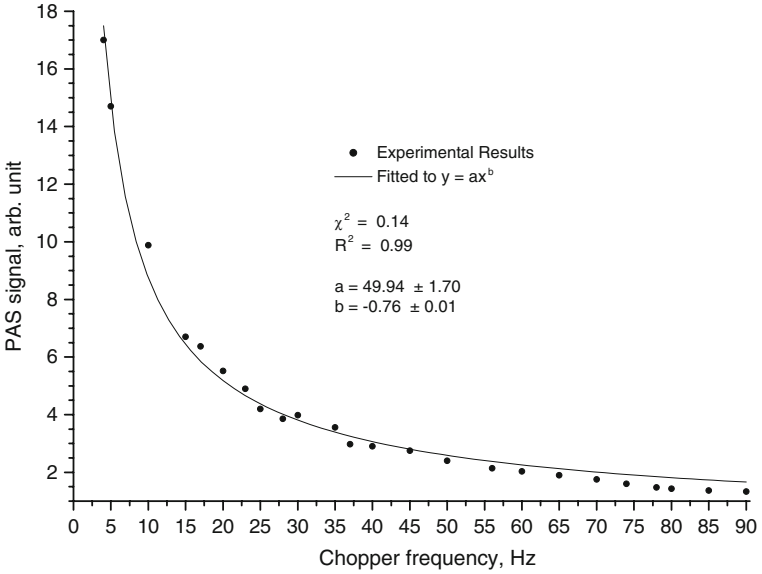

Fig. 4 Photoacoustic signal (in relative unit) of healthy (treated) Acai seed (D2). *Solid line* represents a least-squares fit to the data

Table 1 Influence of modulation frequency on thermal diffusion length and thermally active layer

Chopping frequency ω (Hz)	Thermal diffusion length (μm)	Thermally active layer (μm)
5	244.6	1536.2
10	173.0	1086.3
30	99.8	627.1
50	77.3	489.4
80	61.1	383.7
200	38.6	242.4
550	23.3	146.3
700	20.6	129.3

Figures 3 and 4 (insets) depict the fitting results. It is clearly observed that the exponent "*b*" for infected seeds is lower than that for the healthy ones. The difference is about 10 % for the two samples investigated. At the present time, there is no evidence of such a difference for other infected samples. In fact, because the thermal decay is an intrinsic property of the material, each material will have a different "*b*" exponent. This will be one of the issues for future investigation.

In the second approach (wavelength scanning), the PA signals of infected and healthy Acai seeds were recorded by varying the wavelength of excitation radiation in the interval from 250 nm to 1000 nm (the signal below 300 nm represents noise) while

Table 2 Influence of modulation frequency on thermal diffusion length and thermally active layer

Chopping frequency ω (Hz)	Thermal diffusion length (μm)	Thermally active layer (μm)
5	178.9	1123.4
10	126.5	794.3
30	73.0	458.4
50	56.5	354.8
80	44.7	280.7
200	28.2	177.6
550	17.0	106.7
700	15.1	94.8

keeping the chopper frequency constant at 10 Hz in order to maximize the signal-to-noise ratio. This is not easy to understand because, first of all, 10 Hz is a sub-harmonic of the supply line at 60 Hz. However, the lock-in amplifier is used to solve the problem since the first harmonic is the only one measured. Additionally, the chopper frequency in PAS should be chosen so that the thermal diffusion length must be shorter than the inverse of the optical absorption coefficient. This would depend on the thermal diffusivity as well. However, as is clear from Fig. 5, the obtained spectra are of reasonable quality, which indicates that a good modulation frequency was chosen. Figure 5 shows characteristic absorption bands for the healthy (treated, D2) and infected (D1 and D3) Acai seeds. In PAS, the characteristic peak patterns and signal strengths constitute a basis for differential diagnosis of a disease. Characteristics peaks and bands were observed in the range from 650 nm to 900 nm for the infected samples D1 and D3, and can be ascribed to organic compounds with carboxylates and amines (functional groups) forming the typical metabolic structures of the fungus. It can be observed that the PA signal-to-noise ratio is higher for the sample with fungi scraps when compared with the normally infected sample D3. This is associated with the metabolic signal of the fungus itself, which has in their compositions the carboxylates and amines groups. On the other hand, the healthy sample spectrum in Fig. 5 shows structures in the range from 650 nm to 900 nm which can be ascribed to carboxyl moisture present in the natural oils used to treat the Acai seed.

Even though most biological compounds have distinct absorption patterns in the mid-infrared region, the surface characteristics of seed-like discoloration caused by fungi are generally detectable in the visible range. Hence, the relatively low PA signal of infected seeds when compared with the high PA signal of healthy (treated) ones in the 650 nm to 900 nm band as depicted in Fig. 5 could be attributed to a disorder of compounds. It is probably due to polyphenolic compounds [21], responsible for the seed shell pigment in the course of necrosis causing death of cells and changes of color (from beige to brown) [22]. Since the PAS technique is a reliable tool for detecting fungus activity in seeds, it would be very useful to employ it along with the chromatographic technique in the identification of these compounds. Finally, in the

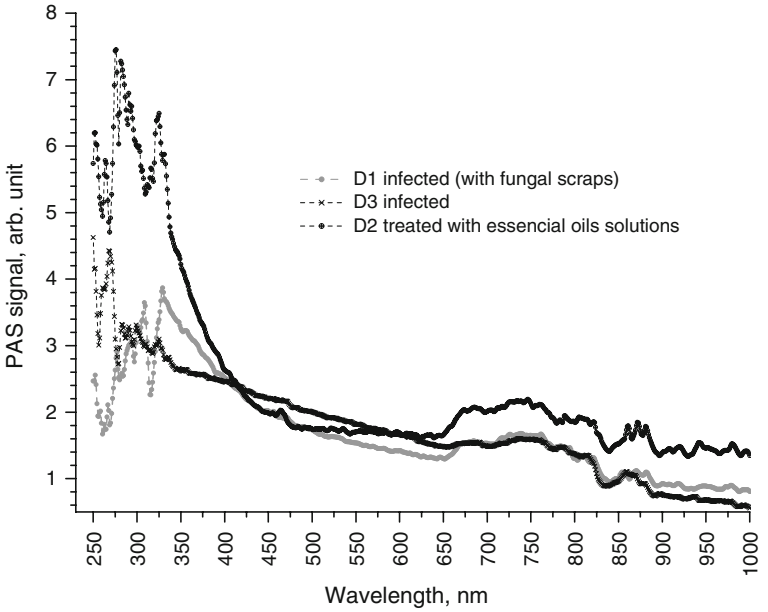

Fig. 5 Photoacoustic signal (in relative units) of healthy and infected Acai seeds as a function of radiation wavelength (wavelength scanning). Superior PA spectral curve is for sample D2 (treated), intermediary PA spectral curve is for sample D1 (with fungus scraps), and inferior PA spectral curve is for sample D3 (fungus infected)

wavelength interval from 300 nm to 350 nm (UV region), the PA signals of infected Acai seeds are smaller than those for healthy ones. That is, the fungus activity modifies the UV absorption property of the Acai seeds.

5 Conclusions

Advantages of PAS include the possibility of obtaining comparable spectra for fungus infected and healthy Acai seeds. They are the basic material of handcrafts such as collar, necklaces, ear rings, among others and regarded as potentially hazardous to humans if not properly treated. In addition, the technique is sensitive, precise, non-destructive, and of low cost. We have demonstrated that PAS can be of great help to the handcraft industry since it can monitor fungi activity by depth-profiling. In this case, the PA signal is very sensitive to the fungal activity in Acai seeds in that the more active, the higher the signal. Our study describes the importance of PAS as a tool to diagnose any fungal disease in seeds and this study at the seed level represents an effort toward a rapid, sensitive, and economical detection method for seed certification standards.

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