Diagnosis and study of fungal diseases of wheat by photoacoustic spectroscopy

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The photoacoustic spectra of healthy wheat leaves and wheat leaves infected with leaf blight caused by Alternaria tritici and with Helminthosporium sativum and with loose smut (Ustilago-nuda-tritici syn. U. segetum (new name)) were recorded in the frequency range of 5 to 400 Hz, keeping the wavelength of excitation radiation constant by using He–Ne laser, to ascertain the depth of penetration of fungal infection within the leaf. The photoacoustic spectra for wheat leaves, moderately and severely infected with leaf-blite disease were recorded abaxially, while the spectra for wheat leaves infected with loose smut were recorded abaxially as well as adaxially. The photoacoustic spectra were also scanned for the healthy leaves of wheat genotypes, EKBSN-1 and FBPEM-2.

The principle of photoacoustic spectroscopy (PAS) was discovered by Alexander Graham Bell1. The fraction of incident chopped radiation, when absorbed by the sample, raises the molecules of the sample from the ground electronic state to the excited electronic state and these excited molecules relax to the ground state through the non-radiative de-excitation, i.e. periodic heat emission. This periodic heat emission produces varying pressure, which is detected as photoacoustic signal. The theoretical and experimental explanation of PAS was developed by Rosencwaig and Gersho2. This technique provides unique possibilities for both applied and basic research. No special sample preparation is required to obtain photoacoustic spectra. Photoacoustic signal allows differentiation between the sample characteristics in different depths, i.e. depth profiling, which is determination of vertical pigments distribution in a leaf sample at different sites, i.e. photoacoustic imaging3.

In the present work, the photoacoustic spectra of wheat leaves infected with leaf-blite (Alternaria spp.) and loose smut (Ustilago-nuda-tritici) were studied and compared to the corresponding spectra of healthy wheat leaves. We have also recorded the photoacoustic spectra of healthy leaves of two wheat genotypes, EKBSN-1 and FBPEM-2, to obtain information relative to photosynthesis within the leaves4.

Photoacoustic spectra were recorded using 632.8 nm radiation of 10 mW linearly polarized He–Ne laser. This radiation passed through a mechanical chopper. This modulated radiation was focused onto the sample compartment of an indigenous photoacoustic cell (2.0 cm diameter, depth) provided with a sensitive gas microphone5. The duration of light pulses and the dark period in between were equal. Photoacoustic spectra were recorded by varying the chopping frequency from 5 to 400 Hz. The signal was amplified by a preamplifier and then processed by a lock-in-amplifier. The lock-in-amplifier, after comparing the reference signal from the chopper and the bunch of signals or noise coming from the various manual assemblies like preamplifier and environment, etc. through the photoacoustic cell, locks the actual signal from the sample and finally amplifies this locked signal millions of times. The time constant of the lock-in-amplifier was kept at 30 s for all measurements.

The photoacoustic spectra of healthy leaves and the leaves infected with loose smut were recorded on abaxial and adaxial leaf surfaces in order to ascertain the depth of pathogen penetration. Great care was taken to minimize the damage and dehydration of leaves used in the experiments.

We prepared a soil mixture containing soil, sand and FYM (farmyard manure) in 1:1:1 proportion. The soil mixture was filled in 29 pots (diameter 9”). The pots were watered and kept in a glasshouse at the Centre of Advanced Studies in Plant Pathology of this university. After 3 days, 10 seeds of wheat variety UP-2382 were sown in each pot, at 5-cm depth. Each pot was covered by a plastic bag to maintain sufficient moisture within it, till the germination of seeds. After germination, plastic bags were removed and the pots were watered daily. After 30 days, about 1 g of urea was added to each pot.

Five pots were kept as control. Inoculation of plants in the remaining 24 pots was done by spore suspension causalagent of leaf-blite in wheat, Alternaria spp. containing 10⁶ conidia (spore)/ml of suspension, with the help of an atomizer.

The wheat leaves (Triticum aestivum L.) infected by leaf-blite pathogen (Alternaria) of various intensities as described above were collected in a polythene bag from the glasshouse and brought to the laboratory for recording the photoacoustic spectra.

About 2 cm² area of the leaves (healthy, moderately infected and severely infected) was cut and enclosed in the photoacoustic cell for recording the photoacoustic spectra. The experimental conditions were the same for each leaf. The experiment was repeated thrice.

Two different approaches can be used for recording the photoacoustic spectra of diseased leaves. Firstly, the modulation frequency is varied and wavelength of the exciting radiation (He–Ne laser) kept constant. This

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technique is called depth profile analysis. Depth profile analysis may be used for in vivo detection of pigments and for testing intactness under changing environmental conditions. One principle of this technique is clearly demonstrated in the photoacoustic spectra of a leaf of *Tradescantia*, by Buschmann and Prehn. The thermally active layer, \( L_a \), is increased by decreasing the modulation frequency within the sample. This means that for a leaf at higher modulation frequency, the peaks of anthocyanins contained in the epidermis are detected. By lowering the modulation frequency, the chlorophyll of the mesophyll below the epidermis contributes to the photoacoustic signal. Consequently, a chlorophyll absorption peak in the red-light region of the spectrum appears. The measurement of a restricted depth of the sample makes it also possible to monitor the pigment characteristics of the two sample sides, separately. While increasing the modulation frequencies into ranges of several hundred Hz, reflection characteristics of the deeper layers may become predominant. Under these conditions, absorption of light reflected from the mesophyll induces a photoacoustic signal in the outer epidermis layer.

In the second approach, the photoacoustic spectra can be recorded by varying the wavelength of the excited radiation (Xe-arc lamp) and keeping the modulation frequency constant. This is called wavelength scanning and is expected to give characteristic peaks (bands) due to synthesis of new molecules or particular nutritional disorders such as those involving zinc, sulphur, etc. during the course of disease development in plants.

In the present work, we have used the first approach and have recorded the photoacoustic spectra by varying the chopping frequency from 5 to 400 Hz.

The leaves of wheat plants showing inflorescence infected with loose smut disease caused by *Ustilago nuda-tritici*, a fungus, and healthy leaves were collected in polythene bags from G1 plot of Wheat Pathology Block at Crop Research Centre of this university and brought to the laboratory.

The leaves of two wheat genotypes, EKBSN-1 and FBPFM-2, were taken from Crop Research Centre of this university. Again the photoacoustic spectra of these leaves were recorded by the procedure described above.

In order to have differential spectra of healthy and infected wheat leaves, the photoacoustic spectra of healthy leaves were compared to those of moderately infected (small leaf spots showing yellowing and destruction of tissues) and severely infected (longer leaf spots with straw-coloured centre and yellow halo around the spots) leaves.

The photoacoustic spectra of healthy leaves and those moderately and severely infected by leaf-blight pathogen were recorded by varying the modulation frequency from 20 to 400 Hz. The excitation radiation used was 632.8 nm He–Ne laser. Photoacoustic spectrum of abaxial leaf surface (upper surface of the leaf facing the incident radiation) is shown in Figure 1. The observed spectral characteristics were markedly different for each leaf, especially between 50 and 240 Hz. At lower frequencies, the strength of photoacoustic signals from moderately and severely infected leaves was comparable, while the healthy leaf exhibited a relatively weak photoacoustic signal. At higher frequencies (240 to 400 Hz), photoacoustic spectra of moderately infected and healthy leaves have similar characteristics, whereas the strength of the photoacoustic signal of severely infected leaves was still higher in this range also.

The leaf-blight fungi have a biotrophic relationship with their cereal hosts. The host cells show autolytic response, i.e. the cytoplasmic organelles slowly disappear and the cells become highly senescent during the course of severe infection.

In severely infected leaves, there is the disappearance of chlorophyll due to disorganization of chloroplast grana in wheat leaves.

Therefore, it is possible to measure the extent of disease by means of photoacoustic analysis, to detect distribution of photosynthesis pigments in diseased leaf tissues at various depths.

Depending upon the modulation frequencies used, the depth profile analysis of the leaves showed three distinct phases. Phase I includes modulation frequencies between 20 and 100 Hz, phase II between 100 and 240 Hz and phase III between 240 and 400 Hz. The photoacoustic signal had maximum strength in phase I, regardless of whether the leaf used for analysis is healthy or diseased. At lower modulation frequencies, the photoacoustic signal obtained is mainly due to chlorophyll contained in the mesophyll tissues below the epidermis.

Photoacoustic signal strength from the diseased leaves was consistently higher than that of healthy leaf (Figure 1). The possible reasons for the enhancement of

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**Figure 1.** Photoacoustic spectra of healthy wheat leaves and those infected by *Alternaria tritici* and *Helminthosporium sativum* (abaxial view).
the photoacoustic signal in the diseased leaves are as follows: (a) The contribution of heat emission through the non-radiative de-excitation in the diseased leaves is large. Due to destruction of chlorophyll molecules in the diseased leaves, the absorbed photons, not trapped by the primary photosynthetic pigments, contribute to heat emission through non-radiative de-excitation, resulting in a strong photoacoustic signal. On the other hand, the healthy leaves displayed weak photoacoustic signal at all the frequencies due to utilization of photons by the photosynthetic pigments, implying reduced contribution to non-radiative de-excitation. (b) The contribution of heat emission through non-radiative de-excitation in the diseased leaves is higher due to the appearance of other biomolecules as a result of fungal infection. These new biomolecules absorb the photons and do not transfer them to the primary photosynthetic reaction-centre. Therefore, they emit these photons non-radiatively, which leads to enhanced photoacoustic signals compared to those from the healthy leaves.

Our above reasoning is strengthened by the work of Helander et al. and Palaria et al. They recorded the photoacoustic spectra of yellow leaf and demonstrated that the absorption in the UV-visible region is strong, due to the destruction of carotenoids and the chlorophyll giving photoacoustic signal when compared with healthy leaves. Green and Steinfeld recorded the photoacoustic spectra of corn contaminated with fungus (Fusarium moniliforme) and have shown that the strength of photoacoustic signal of contaminated corn is large in comparison to that of healthy corn. The enhancement of photoacoustic signal in infected corn was explained as being due to specific biochemical changes that occur when F. moniliforme attacks the corn. Such type of signal enhancement was also observed by Palaria et al. They have shown that this is mainly due to the damage caused to the mesophyll tissues (i.e. destruction of chlorophyll) and simultaneous synthesis of nuclear and extranuclear RNA, and of cytoplasmic protein in the host cell.

At lower frequencies (40 to 70 Hz), the strength of the photoacoustic signal of moderately infected leaves is lower in comparison to the severely infected leaves (Figure 1). This is because the extent of damage caused to the mesophyll tissue in severely infected leaves was higher than that of moderately infected leaves.

At still higher frequencies (240 to 400 Hz), the photoacoustic spectrum is confined to the epidermis, and necrosis (death of cells and change of colour), present over the leaf surface, is the principal cause of variations in photoacoustic signal. Photoacoustic signals of moderately infected and healthy leaves were nearly similar, but for severely infected leaves, the enhancement in photoacoustic signal is apparently due to the presence of severe necrotic tissues, and change of colour from green to straw because of destruction of chlorophyll and other pigments.

Photoacoustic spectra of leaves from loose smut-infected plants were recorded at modulation frequencies ranging from 5 to 400 Hz. The spectra of healthy and infected leaves were recorded on both abaxial (upper surface of the leaf facing incident radiation) and adaxial (lower surface of the leaf facing incident radiation) leaf surfaces, to ascertain the depth of pathogen penetration (Figure 2a and b). In abaxial mode (Figure 2a), at frequencies between 5 and 200 Hz, photoacoustic signal strength of the infected leaves is higher in comparison to healthy leaves, whereas photoacoustic signal strength of both the leaves at frequencies between 200 and 400 Hz is nearly the same. The above spectral changes may be rationalized as follows:

\[
\mu_s = \left( \frac{2k_s}{\rho c_s C_s} \right)^{1/2},
\]

where \( k_s \) is the thermal conductivity of the sample = 6 mJ cm\(^{-1}\) s\(^{-1}\) °C\(^{-1}\); \( \rho \) is the density of the sample = 0.99 g cm\(^{-3}\); \( c_s \) is the specific heat of the sample = 4.18 J cm\(^{-1}\) s\(^{-1}\) °C\(^{-1}\); and \( \omega \) is the frequency of the chopping.

The length of the thermally active layer \( L_s \) is given by \( L_s = \frac{\mu}{\mu_s} \), where \( \mu \) is the thermal diffusion length. The calculated values of the thermal diffusion length \( \mu \) and thermally active layer \( L_s \) are given in Table 1.

<table>
<thead>
<tr>
<th>Chopping frequency ( f ) (Hz)</th>
<th>Thermal diffusion length (µm)</th>
<th>Thermally active layer (µm)</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>96</td>
<td>602.8</td>
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<tr>
<td>10</td>
<td>67</td>
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<td>17</td>
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<tr>
<td>400</td>
<td>11</td>
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It is clear from Table 1 that up to a modulation frequency of 5 Hz, the signal is coming from the whole thickness of the sample and beyond this frequency, the signal is contributed from different layers of the sample, i.e. the leaf. It is clear from Figure 2a that photoacoustic signal strength was higher in diseased leaf in the
frequency range of 5 to 200 Hz. The explanation for this enhancement of photoacoustic signal is similar to the one given for wheat leaf-blight disease. It is also clear from Figure 1 that in the frequency range of 200 to 400 Hz, the magnitude of photoacoustic signals arising from diseased as well as healthy leaves is approximately similar. It is clear from eq. (1) and Table 1 that when the modulation frequency is increased, the signal starts coming from different layers of the leaf. The strength of the photoacoustic signal of diseased leaves was equal to the photoacoustic signal of healthy leaves at 200 Hz. This shows that up to 15 μm thickness from the upper surface of the leaf, the distribution of photosynthetic pigment is the same in both the leaves. After 15 μm depth from the upper surface within the leaf, the photosynthetic pigments are damaged due to infection in the diseased leaf, as the photoacoustic signal strength is large after this depth in the diseased leaf. If we know the thickness of the leaf, we can calculate up to what depth the pathogen is present within the leaf, by subtracting 15 μm from the leaf thickness. This observation shows that the presence of pathogen is at the lower surface in case of loose smut disease, in contrast to the brown rust disease of wheat, in which the pathogen penetrates from the upper surface of the leaf8). Our observation of movement of pathogen through the lower surface of the leaf is strengthened by the fact that in loose smut, the disease originates from infected seed where fungus is present in the form of dormant mycelium (fungus growth). As the seed germinates after sowing, the fungus also becomes active and moves along with the growing points of plants and in this case the fungus enters into the newer tissues of the leaves from their lower surfaces.

Photoacoustic signal of leaves in adaxial mode is shown in Figure 2b. It is clear from this figure that photoacoustic signal strength of the diseased leaves is higher in comparison to the healthy leaves at all the frequencies, i.e. from 5 to 400 Hz.

In this mode, the diseased portion of the leaf is contributing to photoacoustic signal and giving enhanced signal at every frequency (5 to 400 Hz), as the lower surface is facing the incident radiation. This observation again confirms that in loose smut, the infection is entering from the lower surface of the leaf.

Photoacoustic spectra of healthy leaves of two wheat genotypes, EKBSN-1 and FBPFM-2, were recorded by varying the frequency from 5 to 400 Hz. The photoacoustic spectra of abaxial leaf surfaces are shown in Figure 3. We observed from the spectra that up to the frequency of 160 Hz, photoacoustic signal strength for healthy leaves of wheat genotype EKBSN-1 is higher than the healthy leaves of wheat genotype FBPFM-2. At higher modulation frequencies (greater than 160 Hz), the PA spectra of the two wheat genotypes showed similar spectral characteristics. After a frequency of 160 Hz, the photoacoustic spectra of the two wheat genotypes overlapped.

It is known that in healthy leaves the photoacoustic signal is a combination of photothermal (non-radiative
de-excitation) and photobaric (oxygen evolution) contribution. Therefore, if in a particular plant the photosynthetic rate is high, the oxygen evolution will also be high giving strong photoacoustic signal strength. It is clear from Figure 3 that the photoacoustic signal of wheat genotype EKBSN-1 is stronger compared to that of wheat genotype FBPFM-2, in the frequency range of 5 to 160 Hz. Beyond this frequency range (from 160 to 400 Hz), the two signals overlap. This observation shows that the rate of \( O_2 \) evolution in healthy leaves of wheat genotype EKBSN-1 is higher, giving a stronger photoacoustic signal than that of healthy leaves of wheat genotype FBPFM-2, in the frequency range 5 to 160 Hz. At higher modulation frequency (\( \approx 160 \) Hz), \( O_2 \) evolution becomes homogeneous and the microphone of the photoacoustic cell detects the pulse of heat emission induced by the light pulse of excitation light.

The photoacoustic signal strength of two wheat genotypes is nearly equal above 160 Hz, because in this frequency range (160 to 400 Hz) photoacoustic signal arises only due to the contribution of the heat emission (non-radiative de-excitation), which is equal for both wheat genotypes.

In case of wheat genotype EKBSN-1, greener leaves indicate the presence of higher amount of chlorophyll, resulting in greater photosynthesis which is likely to produce bolder grains, giving higher productivity compared to FBPFM-2. Our results showing higher photoacoustic spectrum for wheat genotype EKBSN-1, also indicate the presence of higher amount of chlorophyll, contribution to greater oxygen evolution or photosynthesis, giving higher strength of photoacoustic signals.

The spectral absorption of severely and moderately infected leaves was higher compared to those of healthy leaves, which suggests that some new biomolecules may be formed within the leaf due to the destruction of photosynthetic pigments. These new biomolecules absorb photons, which results in non-radiative transitions, contributing to stronger photoacoustic signals. In case of healthy wheat leaves, the photons are trapped in the primary reaction centre of photosynthetic pigments. In case of wheat leaves diseased with loose smut, increment in photoacoustic signals, in comparison to that of healthy wheat leaves, can be explained as above. Apart from this, we found that abaxial diseased surface of wheat leaves has high photoacoustic signals up to a frequency of 200 Hz, in comparison to that of healthy wheat leaves. Photoacoustic signals are nearly equal at 200 Hz corresponding to a depth of 15 \( \mu m \) within leaf, which suggests that the distribution of the photosynthetic pigments is nearly equal at this frequency. At depth greater than 15 \( \mu m \), the photosynthetic pigments are damaged due to infection in diseased wheat leaves. This result supports the fact that infection penetrates from lower surface of the leaf. The higher photoacoustic signal at all the frequencies in the spectra for the adaxial mode, confirms the above fact. This is also evidenced by the fact that the adaxial mode has consistently higher photoacoustic signal at all the frequencies.

The strength of photoacoustic signals for wheat genotype EKBSN-1 was higher than that of wheat genotype FBPFM-2 up to a frequency of 160 Hz. Beyond this frequency, photoacoustic signals of the two genotypes overlapped, which suggests that the rate of photosynthesis in wheat genotype EKBSN-1 was higher than that of FBPFM-2.


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