Detection of Glyceollin on the Cellular Level in Infected Soybean by Laser Microprobe Mass Analysis

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Laser microprobe mass analysis was explored as a method for the detection at the cellular level of the phytoalexin glyceollin in soybean cotyledons infected with an incompatible race of *Phytophthora megasperma* f. sp. *glycinea*. For LAMMA® analysis 10 µm freeze microtome sections which were freeze-dried on copper grids were used. The LAMMA spectrum of glyceollin (isomer I) shows a characteristic peak at $m/e = 321$ which can be attributed to M-OH$^+$. This peak was also present in the spectra of infected regions from a cotyledon but was completely absent in the spectra of uninfected tissue. One hundred and fifty LAMMA spectra were taken along a line perpendicular to the border line of infection. A steep rise in glyceollin content toward the infected area was observed. This is the first time that such highly localized glyceollin accumulation has been shown at the cellular level. The results show that LAMMA analysis is suitable for the detection of organic molecules in biological tissues with high lateral resolution.

Introduction

Upon infection with *Phytophthora megasperma* f. sp. *glycinea*, soybean seedlings (*Glycine max* L.) accumulate the glyceollin isomers I-III as major phytoalexins [1, 2] (Fig. 1). When the soybean cultivar Harosoy 63 was inoculated in the hypocotyl with mycelium from either race 1 (incompatible) or race 3 (compatible) of *P. megasperma* f. sp. *glycinea*, it was found that 24 h after infection a considerably higher glyceollin* accumulation had occurred in the incompatible interaction [2]. Differences in glyceollin accumulation between the incompatible and compatible interaction were not apparent before about 14 h after inoculation [2]. In these investigations the glyceollin concentration was determined in a 1-cm- long hypocotyl segment which is estimated to contain about 5×10^5 cells. It was, however, to be expected that differences in glyceollin accumulation between the incompatible and compatible interaction at the actual infection site could occur at an earlier time and could be larger. Yoshikawa *et al.* [3] have determined localized glyceollin concentration in about 250 µm thick freeze microtome sections of soybean hypocotyls which were cut parallel to the infection surface. The analytical method used for glyceollin determination was, however, not accurate enough to give reliable results.

An ideal method should allow the determination of glyceollin (or other phytoalexins) in a few cells, or even single cells, in a given area within a tissue. We have now investigated the possibility that laser microprobe mass analysis (LAMMA®) [4] might be such a method. Under certain preconditions LAMMA makes possible the detection of molecules within a biological matrix with high sensitivity and lateral resolution. These preconditions are: 1. The molecule to be detected must be present in the area of interest in a concentration corresponding to the sensitivity of the instrument. 2. The formation of a simple mass spectrum of the molecule with respect to fragmentation which should at least for one mass peak not coincide with the spectrum of the biological matrix. The appearance of pronounced molecular or quasi-molecular peaks is ideal. 3. For identification and possible quantitative determination of the substrate its spectrum from the tissue sample should coincide with that of the pure substance. This can be achieved under certain conditiones by varying the UV-pulse laser with respect to energy, wavelength etc.

As far as we are aware only few attempts have been made to detect organic compounds by the LAMMA-technique in a biological matrix. The detection of isonicotinic acid hydrazide in mycobacteria [5], of lidocain in an ointment [6], and of a car-

* The term glyceollin is used in this paper for the 3 isomers of glyceollin shown in Fig. 1

Abbrevations: LAMMA, laser microprobe mass analyzer.

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dioactive drug in cardiac tissue sections [7] have been reported. In the third case detection was performed via the labelling with a stable isotope (^{19}F).

We now report on the detection of the phytoalexin glyceollin in single cells of soybean cotyledons which had been infected with *P. megasperma* f. sp. *glycinea* and on glyceollin distribution within the tissue around the infection site.

Materials and Methods

Soybean seedlings. Seeds of soybean (*Glycine max* L. cv. *Harosoy* 63) were obtained from R. I. Buzzell, Harrow, Ontario, Canada. Seedlings were grown in vermiculite and potting soil for 5 days as described previously [8].

Fungal cultures. Phytophthora megasperma f. sp. *glycinea* race 1 was obtained from E. Ziegler, University of Aachen, and was grown as described [8].

Inoculation procedure. Cotyledons from 5-day-old seedlings were wounded as described [9] and inoculated with a suspension of mycelial hyphae in distilled water. The inoculated cotyledons were incubated for 40 h on moist filter paper discs at 100% humidity in covered petri plates at 25 °C in the dark.

Isolation and analysis of phytoalexins. For quantitative determination of the phytoalexins, cotyledons were extracted with ethanol and then the extracts were chromatographed on Sephadex LH-20 and analysed by HPLC as described previously [10]. Pure glyceollin isomer I was obtained by preparative HPLC under the same conditions.

Sample preparation for LAMMA analysis. Infected cotyledons were cut with a razor blade into cubes having a side length of approx. 2 mm. The cubes were embedded in Tissue Tek II O.C.T. Compound (Miles laboratories, Naperville, Illinois, USA) and shock-frozen in melting propane. From this material sections of 10 µm thickness were cut perpendicular to the wound surface with a freezing microtome (Frigocut 2700, Fa. Reichert-Jung, Heidelberg) at −18 °C. The sections were transfered to copper grids that had been coated with Formvar film and treated with polylysine. Samples were then freeze-dried immediately.

LAMMA-analysis. The LAMMA®-instrument has been described elsewhere [4]. Briefly, it represents a combination of a laser microscope with a time-of-flight (TOF) mass spectrometer. The microscope serves for the observation of the specimen to be investigated as well as for focusing an UV-pulse laser beam ($\lambda = 265$ nm, pulse durations 30 ns, power $10^8 - 10^{11}$ W cm^{-2}) onto the specimen (spot size 1 µm in diameter). The action of the laser beam leads to the formation of positive and negative atomic and molecular ions which are detected alternately by the TOF-analyzer (mass resolution up to 1000). The detection limit may reach 10^{-20} g for some elements (sodium, potassium) in an analyzed volume of 1 µm^3.

Glyceollin isomer I (mol. weight 338) was dissolved in ethanol and one drop of the solution was brought onto a Formvar-coated copper grid where the solvent was evaporated.

Results and Discussion

For this investigation we used soybean cotyledons since the soft hypocotyl tissue caused greater difficulties in the preparation of the freeze microtome sections. Forty hours after infection with the incompatible race 1 of *P. megasperma* f. sp. *glycinea* one cotyledon of the soybean cultivar Harosoy 63 contained 1.8 µmol glyceollin which corresponds to 9 µg per mg dry weight. The glyceollin isomers I – III (Fig. 1) were present in a ratio of 3 : 1 : 1. In addition 0.6 µmol of 3,6 a, 9-trihydroxypterocarpan [11] was present.

For LAMMA analysis 10 µm thin freeze microtome sections which had been freeze-dried on copper grids were used. To obtain optimal mass spectra

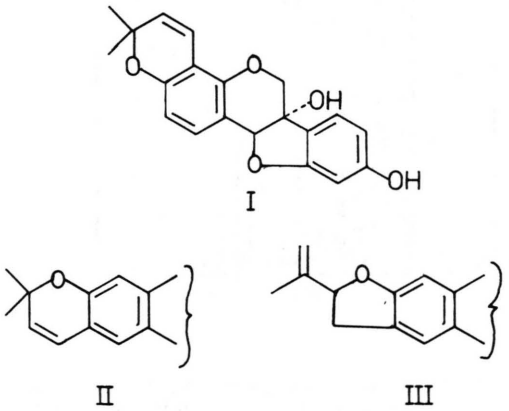

Fig. 1. Structures of the 3 glyceollin isomers which accumulate in infected soybean cotyledons.

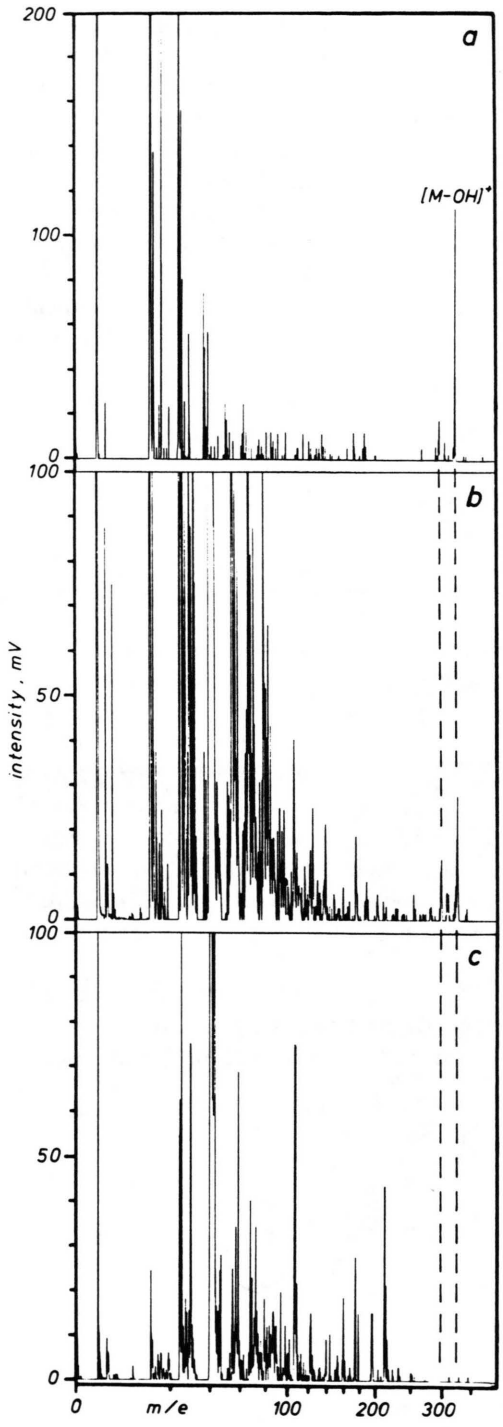

Fig. 2. LAMMA spectra of glyceollin isolated from infected cotyledons (a) and of microvolumes in infected (b) and unifected (c) cotyledon tissue. The dashed lines indicated pronounced fragment ions of glyceollin.

a maximum laser power of about 10^{11} W cm^{-2} had to be used. This high energy caused damage in a tissue area much larger than the diameter of the laser beam. Although this damage occured tim-delayed with respect to the measurements, it prevented the high lateral resolution of the LAMMA-instrument from being fully utilized.

Typcal mass spectra of glyceollin and of the infected and uninfected regions of a cotyledon are shown in Fig. 2 a – c. The LAMMA spectrum of glyceollin shows a charcteristic peak at $m/e = 321$ which can be assigned to M-OH$^+$. This peak together with additional peaks between $m/e = 298$ and 321 are also seen in the spectrum of the infected region, whereas they are completely absent in the spectrum of the uninfected area. Besides the glyceollin peaks further differences can be noted between the spectra of the infected and uninfected areas. These could be due to further differences in the chemical composition and/ or to different properties of the tissues leading to variations in the laser-sample interactions.

The glyceollin concentration (in arbitrary units) along a line perpendicular to the border line of infection is shown in Fig. 3. A steep rise in glyceollin content toward the infected area is apparent. This is the first time that such strictly localized phytoalexin accumulation has been shown on the cellular level.

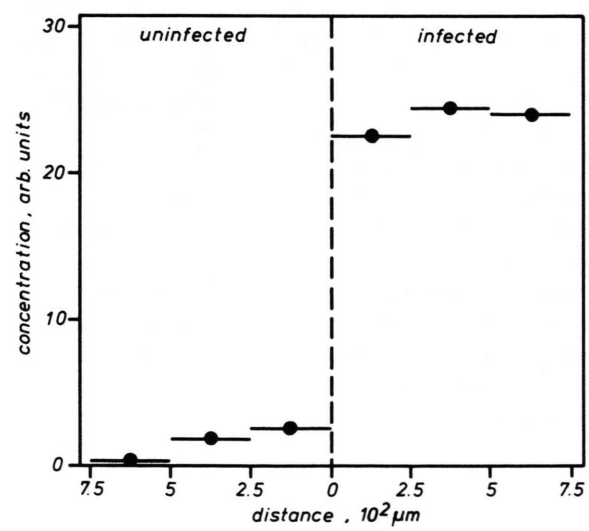

Fig. 3. Glyceollin content (arbitrary units) in infected cotyledons along a line perpendicular to the border line of infection. Each measuring point is an average of 25 LAMMA spectra along a distance of 250 µm. Standard deviation ±7.5 units.

Future investigations will be concerned with the quantitative determination of cellular glyceollin concentrations. Furthermore, the quality of the freeze microtome sections has to be improved and the investigations should be extended to the hypocotyl, the natural infection site of *P. megasperma* f. sp. *glycinea.*

Since the detection limit of the LAMMA instrument was not even fully utilized in our case, one can be hopeful that this method could be a promising approach for other cases in which organic molecules have to be traced in biological tissues with high lateral resolutions.

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