^{13}C NMR Spectra of Plant Pathogenic Fungi

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^{13}C NMR spectra of intact mycelia of *Pyricularia oryzae*, *Botrytis cinerea*, and five species of *Pythium* were measured. Sharp signals assigned to trehalose, mannitol and triglycerides in the cytoplasm were observed in the spectra of *P. oryzae* and *B. cinerea*. (1 $\rightarrow$ 3)- β -Glucan and triglycerides were detected in all species of *Pythium* examined, and trehalose was observed in *P. intermedium*, *P. ultimum* and *P. spinosum*. In addition, glucose was detected in the latter two species. No remarkable signals from proteins, nucleic acids, membrane lipids or cell wall polysaccharides were observed.

Recently NMR spectroscopy has been applied to the fields of biochemistry, physiology and medicine. One well-known application is ^{31}P NMR of intact cells and organs.¹⁾ Energy metabolism in living cells can be observed by following changes in the ^{31}P NMR spectra due to ATP, ADP and sugar phosphates. It is possible to estimate the pH of the matrix of the cytoplasm or organelles by examining the chemical shift of inorganic phosphate. Various samples, microorganisms, cultured cells and mammalian organs, have been studied by ^{31}P NMR. Another application is ^1H NMR, which is being tested for cancer diagnosis using the relaxation time of water to detect tumors.^{2~4)} Lastly ^{13}C NMR with ^{13}C -labeled substrates has been used to study the fermentation processes of *E. coli* and yeast.^{5~6)}

The advantage of NMR is that information on compounds accumulated in the cell can be obtained without any specific preparation. This nondestructive technique is expected to give a new viewpoint in the study of physiological phenomena. However, the major difficulty is that it requires relatively large amounts of samples to obtain clear signals.

In this study we examined the natural-

abundant ^{13}C NMR spectra of intact mycelia of several plant pathogenic fungi; *Pyricularia oryzae*—pathogen of rice blast disease, the most serious disease of rice, *Botrytis cinerea*—pathogen of gray mold disease that attacks vegetables growing in greenhouses, and *Pythium spp.*—a genus of soil-borne infectious Phycomycetes. The compounds responsible for the observed signals in the spectra were identified.

MATERIALS AND METHODS

Strains. The *P. oryzae* strain was isolated from rice at Hokuriku Agricultural Experimental Station in Niigata Prefecture. *B. cinerea* used was isolated from tomato at Vegetable and Ornamental Crops Research Station in Mie Prefecture. The *Pythium* species were isolated from strawberries in Shizuoka Prefecture.

Culture of fungi. *P. oryzae* and *B. cinerea* were cultured on a rotary shaker at 26.5°C for periods of 1 to 3 days in PY-medium consisting of 2.4% dehydrated potato dextrose broth (Difco) and 0.5% yeast extract (Difco). The cultures all reached the stationary phase after 2 days' incubation. Each *Pythium* species was cultured without shaking at 26.0°C for 3 days in PY-medium till the colonies became about 10 cm in diameter.

^{13}C NMR spectra measurement. Mycelia of each fungus

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were collected by filtration and washed three times with distilled water. Two grams of washed mycelia was packed in the bottom of a 10 mm ϕ sample tube, and the ^{13}C NMR spectra were measured at room temperature with a JEOL FX-90Q spectrometer. The spectral width was 5000 Hz, and the spectrum was obtained with a 22 sec (90°) pulse width with proton-noise decoupling and was accumulated 4500 times with a repetition time of 0.8 sec. 8 K data points and a 1.2 Hz broadening window were employed. The resonance of D_2O in a capillary tube inserted in the center of the sample was used for the magnetic field lock. The signal of dioxane (67.4 ppm) was used for the chemical shift standard.

The ^{13}C NMR spectra of the precipitates obtained according to the procedure in Fig. 2 were measured in the same way as for the mycelia. For liquid samples we used a 45° pulse with a 2.8 sec repetition time, and adequate accumulation was determined by the concentration of the contents.

The peaks in the spectra were assigned to the carbons in the compounds in the mycelia. Sugars obtained in the aqueous phase by the method in Fig. 2 and the secreted sugars obtained by soaking of mycelia in distilled water were identified by GC-MS analysis after trimethylsilylation.

RESULTS

^{13}C NMR spectrum of *P. oryzae*

The natural-abundant ^{13}C NMR spectrum of mycelia of *P. oryzae* in the early stationary phase is shown in Fig. 1. The peaks were clearly separated, so compounds could be identified on the basis of their chemical shifts.

The assignment of these peaks was as fol-

lows; 14.7 ppm to alkyl methyl, 21.0~34.5 ppm to methylenes, 128.7 ppm and 130.5 ppm to sp^2 carbons and 172.5 ppm to carbonyl, which indicates the existence of unsaturated fatty acids. The signal of the carbonyl carbon appeared upfield of a free acid signal (for example, 180.3 ppm for linoleic acid), so it was assumed to be in an ester form. Signals between 61.5 ppm and 94.1 ppm are indicative of CH_2OH (about 60~64 ppm), CHOH (about 69~78 ppm) and OCOH (about 90~105 ppm) in sugars. Comparison with the chemical shifts of pure sugars revealed that these signals coincided with those of α,α -trehalose (61.5 ppm C-6, 70.7 ppm C-4, 72.0 ppm C-5, 73.1 ppm C-2, 73.6 ppm C-3, 94.1 ppm C-1) and mannitol (64.3 ppm C-1, 70.7 ppm C-2, 72.0 ppm C-3). GC-MS analysis of the aqueous fraction in Fig. 2 indicated the presence of trehalose and mannitol, and among the substances secreted from mycelia into water, these two saccharides were also detected by GC-MS.

Localization of trehalose, mannitol and fatty acids

Cell fractionation of *P. oryzae* was carried out according to the procedure shown in Fig. 2, and the spectrum of each fraction was measured. The precipitate of the cell homogenate centrifuged at $15,000 \times g$ contained cell

FIG. 1. ^{13}C NMR Spectrum of *Pyricularia oryzae* in the Early Stationary Phase.

f, fatty acid; m, mannitol; t, trehalose.

FIG. 2. Fractionation of Mycelia of *P. oryzae*.* 0.05 M phosphate buffer (pH 7.2), 5 mM MgCl_2 , 0.5 mM EDTA FIG. 3. ^{13}C NMR Spectra of Cell Fractions of *P. oryzae*.

A, aqueous phase; B, chloroform phase; C, precipitate at $15,000\times g$; D, precipitate at $150,000\times g$ (microsomal fraction). c, glycerol moiety in triglyceride; f, fatty acid moiety of triglyceride; g, glucose; m, mannitol; p, *N*-methyl of phosphatidylcholine; t, trehalose.

walls, nuclei, mitochondria, golgi bodies *etc.*, and that at $150,000\times g$ was the microsomal fraction. The supernatant at $150,000\times g$ was the soluble fraction, and was extracted with chloroform and divided into two fractions; the

aqueous phase and the chloroform phase.

The signals of trehalose and mannitol were found only in the aqueous phase (Fig. 3A), suggesting that these two sugars had been present in the cytoplasm. Glucose was also

found in this fraction, but it was probably a degradation product of trehalose generated in the fractionation process, for it was not observed in intact cells.

The sharp signals of the fatty acids in the spectrum of the intact mycelia were found in the chloroform phase spectrum (Fig. 3B). In addition, the peaks at 63.2 ppm and 70.1 ppm corresponding to C-1 and C-2 of glycerol in the ester form were observed in this spectrum. No obvious signals were found in the spectrum of the precipitate at $15,000 \times g$ except for the methylene cluster which might be derived from lipid in nuclei and other organelles (Fig. 3C).

The constituent membrane lipid was found in the microsomal fraction spectrum (Fig. 3D). The line width was broad, but the peak at 54.9 ppm was assigned to $-\text{N}^+(\text{CH}_3)_3$ of phosphatidylcholine. Based on these results it is suggested that the lipid in the spectrum of intact cells was not the phospholipid in the membrane but triglycerides in the soluble fraction.

Spectral changes of P. oryzae during growth

Figure 4 shows the spectral changes of *P. oryzae* during growth. At the logarithmic phase (Fig. 4A), the signals of trehalose and

FIG. 4. ^{13}C NMR Spectra of *P. oryzae* at Different Growth Stages.

A, logarithmic phase; B, early stationary phase; C, late stationary phase.
a, amino acid; f, fatty acid moiety of triglyceride; m, mannitol.

FIG. 5. ^{13}C NMR Spectrum of *Botrytis cinerea*.

f, fatty acid moiety of triglyceride; m, mannitol; t, trehalose.

mannitol were found, but the triglyceride signals were small and formed a vague cluster. The signals at 40.4, 41.5, 55.4 and 157.5 ppm were probably due to amino acids, such as arginine. The amino acids disappeared at the early stationary phase (Fig. 4B), and triglycerides increased. At the late stationary phase (Fig. 4C), the triglyceride signals became increasingly stronger and those of mannitol decreased to undetectable levels.

^{13}C NMR spectrum of *B. cinerea*

The ^{13}C NMR spectrum of *B. cinerea* in the early stationary phase is shown in Fig. 5. This spectral pattern, indicative of trehalose, mannitol and triglyceride, was similar to that of *P. oryzae* at the same growth stage.

^{13}C NMR spectra of *Pythium*

The ^{13}C NMR spectra of five species of

Pythium are shown in Fig. 6. The signals of triglycerides and a sugar whose anomeric carbon was resonant at 103.5 ppm were common among these species. As the signal of the anomeric carbon was down field from a monosaccharide, the common sugar was assumed to be an oligo- or polysaccharide. This sugar was obtained by soaking of mycelia of *P. angustatum* in distilled water for 24 hr and its constituent sugar was analyzed by GC-MS after hydrolysis and trimethylsilylation. The only monosaccharide detected was glucose and the chemical shift of each carbon in the common saccharide showed good correspondence to that of β -laminaribiose. Hence we presumed it was (1 $\rightarrow$ 3)- β -glucan. The narrowed line width of the signals of this glucan suggested that it was not a cell wall component but stored in the cytoplasm.

Trehalose was detected in the spectra of *P. intermedium*, *P. ultimum* and *P. spinosum*. Glucose was also found in the latter two species.

DISCUSSION

In the ^{13}C NMR spectra of fungal mycelia we observed the signals of triglycerides and sugars accumulated in the cytoplasm. The sugars detected in the spectra of *P. oryzae* and *B. cinerea* were trehalose and mannitol, while (1 $\rightarrow$ 3)- β -glucan was common in the *Pythium* spp. with the additional presence of trehalose and glucose in some of them. Amino acids were found only in the logarithmic phase of *P. oryzae*.

Intermediates of active metabolic pathways could not be detected, which suggests that their amounts were small and their lifetimes were short in comparison with the timescale of the measurement of the NMR spectra. The signals of protein, nucleic acid and lipid in the membrane were not observed in the spectra of intact mycelia. In addition, signals of cell wall components did not appear, though Matsunaga *et al.* reported broad peaks of galactan from the cell wall in the ^{13}C NMR spectrum of *Penicillium ochro-chloron*.⁷⁾

FIG. 6. Sugar Regions of ^{13}C NMR Spectra of Five Species of *Pythium*.

A, *P. intermedium*; B, *P. ultimum*; C, *P. angustatum*; D, *P. myriotylum*; E, *P. spinosum*. b, C-3 of glucose in (1 $\rightarrow$ 3)- β -glucan; b' C-1 of glucose in (1 $\rightarrow$ 3)- β -glucan; g, C-1 of α -glucose; g', C-1 of β -glucose; t, C-1 of trehalose.

Peaks b and b' indicate the presence of (1 $\rightarrow$ 3)- β -glucan, peaks g and g' that of glucose, and peak t that of trehalose.

Usually the signals of these large molecules can not be observed because their line width is broadened due to their low mobility.

Since the sugars and lipids detectable in ^{13}C NMR spectra of intact mycelia are involved as substrates or intermediates in energy production and biosynthesis, spectral changes on fungicide application are expected to reflect the action of the drug on metabolism and biosynthesis. Changes in the biosynthetic pathways will be detected in the NMR spectra if an intermediate accumulates in the matrix of the cytoplasm or an organelle. In such cases, NMR will be a powerful technique for the investigation of the mechanism of action of a fungicide.

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