Formation of Lignin-like Substance in the Root Tissues of Japanese Raddish Plant Infected by Downy Mildew Fungus*

Yasuji ASADA** and Isao MATSUMOTO**

浅田泰次**・松本 勲**： ベと病菌に感染したダイコン根組織内における
リグニン様物質の生成

Abstract

A lignin-like substance (LS) has been found in roots of Japanese radish infected by *Peronospora parasitica*. From infrared and ultraviolet absorption spectra, methoxyl content, and paper chromatograms of the degradation products, it was confirmed that LS from infected roots is different from wood lignin. This substance was not found in healthy parenchyma. Living tissues alone were capable of forming this substance. Histochemical reactions by phloroglucinol-HCl and Loele's reagents revealed that high peroxidase activity was located around the lignified cell walls. All cells in the sections were alive judging from plasmolysis except those on the cut surface. However, we could not determine exactly whether the lignification of cell walls had taken place before or after the fungal attack. Lignification in cell walls of parenchyma was incomplete even when the cells were infected.

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Introduction

Nutritional physiology of the obligate parasite and the defense reaction of a host to the parasite are interesting problems to both the plant pathologists and biochemists. In the previous paper¹⁾, we reported the formation of lignin in the root tissues of Japanese radish affected by *Alternaria japonica*. In order to compare the reactions between facultative saprophyte and obligate parasite, we studied the lignin formation in the root infected by *Peronospora parasitica*.

Material and methods

Inoculation Well-developed roots of about 3-month-old Japanese radish (*Raphanus sativus* L. var. *hortensis* Backer) were obtained from the market. The roots (ca. 5 cm in diam.) were aseptically cut into slices about 1 cm thick. The slice were inoculated with conidia of *Peronospora parasitica* Pers. ex Fr. (*P. brassicae* Gäum.)¹¹⁾. The conidia were collected from infected leaves by keeping them in moistened petri dishes for one night. Inoculated slices were incubated in moistened petri dishes at 20°C under 110 lux illumination from white fluorescent lamps.

Histochemical reagents and plasmolysis Slices of parenchyma, about 50 μ thick were pre-

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** College of Agriculture, Ehime University, Matsuyama, Japan. Laboratory of Plant Pathology, Contribution (N.S.) No. 3. 愛媛大学農学部

pared by a freezing microtome. Phloroglucinol-HCl reagent was added to slices to identify the presence of lignin. Peroxidase was identified by Loele's reagent⁵⁹. For observing plasmolysis, hand-sectioned slices were dipped into 0.4 M KCl for 30 min.

Isolation and quantitative determination of lignin Twenty g of powder (30-mesh) were prepared from dried slices. This powder was suspended in 1 l of hot water for 12 hr and filtered twice. The residue was extracted with 2 l of 0.5% ammonium oxalate at 85°C for 30 hr and filtered, followed by extraction of the residue again with 1 l of 2% Na₂CO₃ for 12 hr. After drying the residue at 40°C, the powder obtained was extracted with 100 ml of benzene-ethanol (1:1) at about 80°C for 12 hr. Then the residue was extracted with 200 ml of dioxane containing 3 ml of 12 N HCl at about 90°C for 12 hr. The filtered residue was washed with dioxane. The filtrate and dioxane washings were neutralized with solid NaHCO₃. The filtrate was distilled under reduced pressure and about 50 ml of concentrated solution were added to 400 ml of HCl solution (pH 2), centrifuging at 1,600×g for 20 min. After washing the centrifuged material with 200 ml of water, the pellet was dissolved in 10 ml of dioxane, 400 ml of ether added and the suspension was centrifuged twice at 1,600×g for 20 min. The final precipitate is designated as "dioxane lignin-like substance" (DLS).

Quantitative determination of lignin content was made as follows: Five hundred mg of powder, extracted with benzene-ethanol mixture (mentioned above), were treated with 0.5 g thioglycolic acid in 5 ml of 2 N HCl at about 90°C for 5 hr, and filtered through a glass filter. The residue was extracted with 5 ml of 2% NaOH at room temperature for 24 hr. The filtered residue was suspended in 10 ml of water for a few minutes. The filtrate and water washings were acidified to pH 1.5 with 5 N HCl and centrifuged at 1,600×g for 20 min. The precipitate was washed with 10 ml of water and centrifuged at 1,600×g for 20 min. The pellet was extracted with 10 ml of ether and centrifuged again. The final precipitate was dissolved in 5 ml of N,N-dimethylformamide and the absorbance was determined at 278 mμ. The absorbance of this preparation at 0.05 mg/ml was 0.62. Cut roots and boiled roots served as controls.

Identification of lignin DLS was characterized by infrared (IR) and ultraviolet (UV) absorption spectra, by determination of methoxyl groups, and by paper chromatography of the degradation products. KBr tablets were used for IR absorption determinations. The UV absorption spectra of 0.1 mg/ml DLS were taken in 80% dioxane solution. Methoxyl groups were quantitatively determined by Vieböck-Brecher's method⁷⁰. For paper chromatography, 50 mg DLS were degraded at 180°C for 2 hr in a mixture containing 20 ml of 2 N NaOH and 0.5 ml of nitrobenzene. To remove residual nitrobenzene, the reacted solution was steam-distilled. After acidification to pH 1.5 with 3 N HCl, the solution was continuously extracted with ether for 70 hr. The ether extract was dissolved in 1 ml of 95% ethanol and the solution was applied to sheets of Tôyô filter paper (No. 50). Chromatograms were run in *n*-butanol solvent saturated with 3% ammonium hydroxide. To identify vanillic acid, 1% ethanol solution of quinone chloroimide and 1% Na₂CO₃ were used as a spray after exposure of the filter paper to acetic acid vapor. For vanillin, *p*-hydroxybenzaldehyde, and syringaldehyde, a solution containing 1% 2,4-dinitrophenylhydrazine in 5% HCl was used. Each spot was extracted with 5 ml of 95% ethanol for 24 hr, and the amounts were measured at 306 mμ (for vanillin and syringaldehyde), at 286 mμ (for *p*-hydroxybenzaldehyde), and at 260 mμ (for vanillic acid). The relationship between extraction period and extracted amount of the degradation products obtained from the separated spots of paper chromatogram was shown by the following equation:

$$Y = \frac{X}{0.0111 + 0.0089X} \quad (\text{where } Y: \text{Extracted amount } (\%); X: \text{Extraction period (day)})$$

The absorbances of vanillin, syringaldehyde, *p*-hydroxybenzaldehyde, and vanillic acid at 0.01 mg/ml were 0.74, 0.69, 1.40, and 0.60, respectively.

Results and discussion

Conidia formation on excised sections Table 1 shows number of conidia produced on excised sections. Illumination was found to be necessary for conidial formation. Figs. 1, 4, 5, in Plate I show haustoria, conidiophores and conidia on the sections.

Sites of lignification and peroxidase activity Fig. 1 shows camera-lucida drawings of transverse and longitudinal sections of the Japanese radish root. The roots consist mostly of xylem parenchyma¹⁰⁾. Plate II shows the reactions of infected and noninfected roots to phloroglucinol-HCl and Loele's reagents. Immediately after inoculation, lignin formation was observed only at the cell walls of periderm and vessels. Three days after inoculation, cell walls of parenchyma were found to be lignified. Infecting hyphae were observed near lignified cells. However, we could not observe with precision whether the lignification had taken place before or after the fungal attack. As shown in Figs. B, C, in Plate II, very little lignification was apparent for noninfected parenchyma, and this can be seen at the lower part of the infected cells 1 to 2 days after inoculation. The depth of infection in the sections 3 days after inoculation was about 1.5 mm. Moreover, lignification in the walls of infected parenchyma was incomplete. If the lignification is complete, the hyphae may not attack lignified walls and the infection may then stop. In fact, infected hyphae did not attack previously lignified vessels. (see Fig. 3 in Plate I). Peroxidase activity was also observed histochemically near lignified walls. All cells in these sections except the cut surface were alive as observed by plasmolysis (see Fig. 2 in Plate I). Until 3 days after inoculation, the browning reaction was not observed even at the surface of the sections. The browning reaction appeared about 5 days after inoculation. In these tissues host cells had already died. Therefore, it seems that the browning

Table 1. Number of conidia formed on the excised section of downy mildew-infected Japanese radish roots

Number of conidia per unit area (cm ²) ^{a)}	Incubated days ^{b)}			
	0	5	10	15
I	0	0	243	90
II	0	0	313	155
III	0	0	335	112
Avg.	0	0	297	119

a) Average surface area of inoculated sections is 11.3 cm².

b) Light: 110 lux, Temp.: 20°C.

Fig. 1. Histological illustration of Japanese radish root.

Per: Periderm, Cor: Cortex, Phl: Phloem, Cam: Cambium, Xyl. par: Xylem parenchyma, Ves: Vessel, Xyl. fib: Xylem fiber, Sec. ves: Secondary vessel.

reaction does not occur as a defense reaction of the host but occurs as the result of death of the host. The cell wall of the parenchyma in control sections is not lignified even at the cut surface. Therefore it would appear that lignin formation seems to take place, not by cutting, but by fungal infection. It should be noted that living cells are necessary to form lignin. Since lignin is probably formed from enzymatic dehydrogenation of specific phenylpropane monomers, living cells may be required. DeJong²⁹ reported that peroxidase reaction is strongest in nonlignified tissues, but is absent in lignified xylem throughout all stages of the development of onion root. Higuchi and Ito⁴³ indicated that laccase and peroxidase activities are probably responsible for dehydrogenation. Freudenberg³⁹ suggested the following mechanism for initiating dehydrogenation polymerization of coniferyl alcohol. The coniferyl alcohol (I) is dehydrogenated at phenolic hydroxyl group. A mesomeric radical is formed with limiting structures, such as aroxyl form (II), *p*-quinone methide radical (III), and orthoquinoid form (IV). The combination of

(III) and (IV) gives a quinone methide, which is stabilized by intramolecular prototropy, giving dehydroconiferyl alcohol. Two (III) radicals give a double quinone methide, which undergoes a double prototropy, giving pinorensinol. These substances seem to be components of lignin³⁹.

Thus peroxidases, which are widely distributed in plants, and for which the activity increases following fungal infection, seem to play a role in lignin formation. The extraction and identification of peroxidases should be followed in future studies.

Identification of lignin The UV absorption spectra of DLS are shown in Fig. 2. Curves A and B show absorbances in basic and neutral dioxane solutions, respectively, and C shows the difference ($\Delta\epsilon$) between them. Curve B had a maximum at 278 m μ ; the maximum value of $\Delta\epsilon$ was about 300 m μ .

Fig. 3 shows the IR absorption spectrum of DLS. Absorptions at 3,400, 2,920, and 2,850 cm⁻¹ indicated the presence of C=O, C=C aromatic, and C-H groups, respectively. These results suggested that the isolated substance had the properties of lignin. However, since these spectra are not as well defined as those of wood lignin, some contamination or decomposition during isolation might have occurred. Moreover, since no methods have yet been devised for isolating lignin in the natural state, some chemical alteration may have occurred.

The amount of methoxyl group in DLS is shown in Table 2. This amount is higher than that from

Fig. 2. Ultraviolet absorption spectra and $\Delta\epsilon$ of the isolated dioxane lignin-like substance obtained from inoculated Japanese radish root. A: Basic dioxane solution, B: Neutral dioxane solution, C: $\Delta\epsilon$.

Fig. 3. Infrared absorption spectrum (KBr) of the isolated dioxane lignin-like substance obtained from inoculated Japanese radish root.

Table 2. Amount of methoxyl group in the isolated dioxane lignin-like substance^{a)}

Weight of sample (mg)	Amount of Na ₂ S ₂ O ₃ (ml)	OCH ₃ (%) ^{b)}
4.05	9.6	12.11
3.35	8.2	12.51
3.35	8.8	13.13
		Avg. 12.58

^{a)} The substance was obtained from infected roots 15 days after inoculation.

^{b)} $\frac{N \times 0.0517}{S} \times 100$

where N: Amount of N/100 Na₂S₂O₃ (ml),
S: Weight of sample (mg).

Table 3. *R_F* values and the amounts of the degradation products by alkaline nitrobenzene oxidation of the isolated dioxane lignin-like substance

Product	<i>R_F</i> value ^{a)}	Amount (%) ^{b)}	Formula ^{c)}
Vanillic acid	0.09	2.76	R-COOH
Syringaldehyde	0.29	2.76	R'-CHO
Vanillin	0.35	6.16	R-CHO
<i>p</i> -Hydroxybenzaldehyde	0.44	1.48	R''-CHO

^{a)} Solvent mixture: *n*-Butanol saturated with 3% NH₄OH.

^{b)} The amounts were corrected by the previously mentioned equation.

^{c)} R=Guaiacyl, R'=Sringyl, R''=Hydroxyphenyl.

roots infected by *Alternaria japonica*¹⁾. It seems that phenylpropane monomers for polymerization is different depending on the source. The DLS constituents were next examined. Table 3 shows the *R_F* values and amounts of the degradation products by alkaline nitrobenzene oxidation. Vanillin was highest in amount. Fig. 4 shows the UV absorption spectra of the degradation products. The spectra of vanillic acid, vanillin, and *p*-hydroxybenzaldehyde were consistent with those of authentic compounds. However, syringaldehyde and its authentic compound differed in their spectra. Therefore, the degradation products seem not to contain syringaldehyde. DLS from roots infected by *A. japonica* contained 9.9% of vanillin and 0.4% syringaldehyde. Since higher amount of methoxyl group was observed in DLS from *Peronospora*-infected roots than that from *Alternaria*-infected roots, there may be differences in the DLS constituents.

Quantitative determination of lignin content It was previously shown that the amount of lignin-like substances obtained by thioglycolic acid treatment was more than 10 times that of DLS which was extracted by dioxane¹⁾. Apparently, dioxane did not extract lignin effectively. For the quantitative determination of lignin, thioglycolic acid may be a useful extracting solvent. Fig. 5 shows

Fig. 4. Ultraviolet absorption spectra of authentic compounds and the degradation products obtained from the extraction of paper chromatograms.

A: Authentic compound, B: Degradation product, I: Vanillic acid, II: Vanillin, III: Syringaldehyde, IV: *p*-Hydroxybenzaldehyde.

the amount of LS in roots infected or incubated without fungi. The amount increased following infection. Noninoculated and boiled roots showed no increases during incubation. These results show that lignin formation in roots takes place only in infected living tissues.

Amount of methoxyl group in the isolated dioxane lignin-like substance Table 4 shows the amounts of methoxyl group in DLS from infected or cut roots. The amount of $-OCH_3$ decreased following infection. For comparing

Fig. 5. Changes in the amount of the isolated lignin-like substance in inoculated and noninoculated sections following infection or incubation.

A: Inoculated root, B: Noninoculated root, C: Noninoculated boiled root.

chemical properties of lignin, it should be necessary to determine the amount of $-\text{OCH}_3$. Coniferyl alcohol, synapyl alcohol, and *p*-coumaryl alcohol that have been proposed as precursors of lignins in plants, have 1, 2 and zero molecules of $-\text{OCH}_3$, respectively. Differences in the amount of $-\text{OCH}_3$ in DLS may be the result of differences in the content of precursors. If we represent A; the total amount of LS in infected roots, B; the amount of LS in noninfected roots, X; the calculating amount of $-\text{OCH}_3$ in DLS of infected parenchyma, C; the amount of $-\text{OCH}_3$ in DLS of noninfected roots, and D; the amount of $-\text{OCH}_3$ in DLS of infected roots, the following equation may be taken.

$$\frac{A-B}{A} \times \frac{X}{100} + \frac{B}{A} \times \frac{C}{100} = \frac{D}{100} \quad X = \frac{AD-BC}{A-B}$$

Table 5 shows the results of the calculation. The DLS in infected parenchyma which is freshly formed by the infection seems to be low content of $-\text{OCH}_3$ as compared with that of DLS which is present in vascular bundles and periderms of noninfected roots. Amount of $-\text{OCH}_3$ in Klason lignin of bamboo shoots was proportional to the intensity of the phloroglucinol-HCl reaction⁶⁾. In fact, we have observed histochemically the difference of the color reaction between the cell walls of vascular bundle and of infected parenchyma.

Conclusion

Since an obligate parasite can not survive in an artificial medium, living tissues must be used for the cultivation of the parasite. The use of Japanese radish root and *Peronospora* complex for this purpose was first proposed by Nakamura⁸⁾. Although tissue cultures of the obligate parasite-infected host are desirable, the method is very difficult and the fungal growth is slow. It is important that the forma* used be carefully identified, that samples of the same developmental stage be chosen, and that the material always be cultivated under same conditions. However, the roots used were obtained from the market, because we did not have an experimental field of our own.

Lignification in infected tissues seems to have the following meaning. Since fungi in infected tissues generally remain alive even after the cessation of enlargement of infected area, it is assumed that the infected hyphae are enclosed by a barrier formed in the host tissues. The most effective barrier to hyphal growth may be lignin. However, the lignification on cell walls of infected parenchyma was incomplete. Therefore, the walls are not lignified enough to arrest

Table 4. Changes in the amount of methoxyl group in the isolated dioxane lignin-like substance in response to infection

Amount of OCH_3 (%)	Days after inoculation			
	0	5	10	15 ^{a)}
I	19.77	16.68	13.94	13.13
II	18.87	17.04	14.26	12.11
III	18.17	17.28	14.94	12.51
Avg.	18.93	17.00	14.38	12.58

a) See Table 2.

Table 5. Calculated amount of methoxyl group in dioxane lignin-like substance in infected parenchyma^{a)}

Days after inoculation	Total amount of LS ^{b)}	Amount of LS formed	Amount of OCH_3 ^{c)}	Calculated amount of OCH_3
0	1.06	0	18.93	0
5	1.43	0.37	17.00	11.47
10	2.04	0.98	14.38	9.45
15	3.25	2.19	12.56	9.50

a) All figures in %.

b) Refer to Fig. 5.

c) Refer to Table 4.

* In this experiment, some kinds of Japanese radish (*R. sativus* var. *hortensis* f. *miyashige*, — f. *minowase*, — f. *sempervirens*) were used.

Plate I

- Fig. 1. Haustoria of *Peronospora parasitica* in parenchyma of Japanese radish root.
 Fig. 2. Living cells of infected parenchyma showing plasmolysis. H: Haustoria.
 Fig. 3. Infected hyphae in the section. The hyphae can not attack the lignified vessel. H: Haustoria; V: Vessel.
 Fig. 4. Development of conidiophores of *Peronospora parasitica* on the surface of excised section 10 days after inoculation. H: Haustoria.
 Fig. 5. Conidia and conidiophores of *Peronospora parasitica* on excised section of Japanese radish root.

Plate II

Reactions of root tissues to phloroglucinol-HCl (for lignin) and Loele's (for peroxidase) reagents in response to the infection of *Peronospora parasitica* or the incubation of section.

A: 0 day after inoculation.

B: 1 day after inoculation.

C: 2 days after inoculation.

D: 3 days after inoculation.

a: 0 day after cutting.

b: 1 day after cutting.

c: 2 days after cutting.

d: 3 days after cutting.

Per: Periderm, Cor: Cortex, Phl: Phloem, Cam: Cambium, Xyl. par: Xylem parenchyma, Ves: Vessel.

Thick parts on cell walls show the presence of phloroglucinol-HCl reaction. Shaded and dotted portions show dead cells and the presence of peroxidase activity.

the fungal growth as a mechanical barrier. This fact seems to be due to the following reasons. (i) Since the excised sections used were always kept under the most favorable conditions for the fungal growth, the pathogenicity of the fungus was constantly vigorous; (ii) although high metabolic activity is required for the defense reactions in host plants, the excised sections did not have the complete activity as compared with that of the whole living plants. Moreover, the disease usually occurs not in the root but on the leaves of Japanese radish. Therefore, lignin formation in the leaves infected by the fungus is problematic. The production of phytoalexin-like substances which react to the invaded hyphae is a further problem that should be studied. The barrier hypothesis should be investigated further with special reference to the nature of disease resistance in higher plants.

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和文摘要

べと病菌に感染したダイコン根組織内における リグニン様物質の生成

浅 田 泰 次, 松 本 勲

べと病菌に侵されたダイコン根組織はリグニン様物質を生成する。紫外および赤外吸収スペクトル, メトオキシル基量およびアルカリ分解物のペーパークロマトグラムによると, 本物質は木材リグニンとは性質が異なる。本物質はまた健全柔組織の細胞膜には存在せず, べと病菌の感染を受けた生きた柔組織のみによって生成される。組織化学的観察によると, 木化細胞膜の近辺ではパーオキシダーゼ活性がいちじるしい。褐変は接種あるいは切断3日後の切片ではその表面においてもみられない。これらの組織は切断表面を除いてすべて原形質分離を起こす。しかし柔組織細胞膜の木化は, べと病菌の侵害に先だって起こるのか, 侵害された後の柔組織細胞膜で起こるのかは明らかにできなかった。また侵害柔組織細胞膜での木化は部分的であり, 全細胞膜が木化することはなかった。