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The Nature of Induced Lignin in Carrot (*Daucus carota* L.) Callus Cells Elicited with Hot Water Extracts from Conidia of *Botrytis cinerea*

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Abstract

Carrot callus cells showed a positive reaction to phloroglucinol-HCl after eliciting with hot-water extract from conidia of *Botrytis cinerea*. The positive reaction reflected increases in the content of thioglycolic acid lignin and in the activity of phenylalanine ammonia-lyase. Alkaline nitrobenzene oxidation of thioglycolic acid lignin eliminated both *p*-hydroxybenzoic acid and vanillin. The amount of *p*-hydroxybenzoic acid increased after eliciting with hot-water extract of conidia, but the amount of vanillin did not increase. The only phenolic substance liberated from thioglycolic acid lignin by alkaline hydrolysis was *p*-hydroxybenzoic acid and the amount was also increased by elicitation. The phenolic substances in dioxane lignin were *p*-hydroxybenzoic acid, vanillin, vanillic acid, and syringaldehyde, but only the amount of *p*-hydroxybenzoic acid was increased after elicitation. Phenolic substances liberated from cell wall materials by alkaline hydrolysis were predominantly *p*-hydroxybenzoic acid; *p*-hydroxybenzoic acid in particular increased in cell wall materials from cells elicited. The increased *p*-hydroxybenzoic acid formed ester-bonds via its hydroxyl and carboxy groups to cell wall materials. Induced lignification in cultured carrot callus cells indicated an increase of ester-bonded *p*-hydroxybenzoic acid in lignin precursor or wall polysaccharides rather than *de novo* synthesis of lignin.

Key words: *Botrytis cinerea*, callus culture, carrot, *p*-hydroxybenzoic acid, induced lignin, lignin, phenylalanine ammonia-lyase.

Abbreviations

AIR, methanol insoluble residue; CWM, cell wall material; DLS, dioxin lignin substance; DMSO, dimethyl sulfoxide; *p*-HBA, *p*-hydroxybenzoic acid; HWE of conidia, hot-water extract of conida; LTGA, lignin-thioglycolic acid; MS medium, Murashige-Skoog medium; PAL, phenylalanine ammonia-lyase; SH medium, Schenk-Hildebrandt medium; TMSi derivative, trimethylsilylated derivative; Van, vanillin.

Introduction

Lignins are well-studied phenolic polymers found in the secondary cell wall of plants. These substances are synthesized by enzymic dehydrogenation of the hydroxyl-cinnamyl alcohols such as *p*-coumaryl, coniferyl, and sinapyl. The phenolic composition of lignin varies among plant family and parts of plant (Lewis and Yamamoto, 1990).

Plant cell walls also contain non-lignin phenolic substances, including hydroxycinnamic acid derivatives (Fry, 1982, 1983; Ishii, 1990; Iiyama *et al.*, 1994). These hydroxycinnamic acids and their dimers are known to be directly esterified or etherified for cross-linking to the polysaccharides in the cell walls. Phenolic substances in the cell walls are important factors in the control of the rigidity and extensibility of cell walls (Ishii, 1997; Yang and Uchiyama, 2000a, b), and may be significant in disease resistance reactions (Uchiyama *et al.*, 1983; Farmer, 1985; Barbera and Ride, 1988).

Lignin or lignin-like substances are formed in plant cell walls in response to substances (elicitors) produced by many plant pathogens (Robertsen and Svalheim, 1990; Messner and Boll, 1994). Deposition of lignin in the cell walls has been proposed to be important defense mechanisms of many plants against biotic stress. It has been believed that in-

duced lignification acts as a physical barrier to pathogen invasion and interferes with enzymatic hydrolysis of the cell wall by pathogens (Fry, 1982; Yang and Uchiyama, 2001). Induced lignification is also accompanied by increased activity of phenylalanine ammonia-lyase (Cahill and Mc Comb, 1992), which is involved in phenylpropanoid metabolism, and peroxidase (Jaegher *et al.*, 1985; Cvikrova, *et al.*, 1994), which catalyzes the last step in lignin biosynthesis. These enzymes are also activated, and new types of lignin appear to be formed, in resistant reactions of plants. Root tissues of Japanese radish inoculated with *Pernospora parasitica* produced lignin or lignin-like substances. Asada and Matsumoto (1972) compared lignins from healthy and diseased tissues, and found that they contained predominantly syringyl- and guaiacyl type lignins, respectively.

It is difficult to reproduce host-parasite interaction accurately in rice callus tissues, because these tissues lack physical barriers such as a cutin layer and have diminished R-genes expression relative to whole rice plants (Uchiyama and Ogasawara, 1977; Uchiyama *et al.*, 1984). But plant tissue culture techniques also have many advantages in the study of host-parasite interactions, because fungal pathogens can be cultured together with their host under controlled chemical and physical conditions. Responses of callus tissues to pathogens may be significant for clarifying defense reactions of plants (Heale and Sharman, 1977; Yamada *et al.*, 1993; Nomura *et al.*, 1997).

After treatment of carrot callus cells with conidia, heat-killed conidia, or hot water extracts (HWE) from conidia of *Botrytis cinerea*, a necrotrophic fungus, the surfaces of the callus tissues showed a positive reaction (red color) with phloroglucinol-HCl. This reagent is aldehyde selective, and detects the accumulation of materials called induced lignin or lignin like substances. There are many articles reporting induced lignifications in tissue culture, but the nature of the induced lignin is unclear.

The present work was undertaken to elucidate the nature of lignin-like substances induced in the cell walls of carrot callus after elicitation with hot-water extracts of *B. cinerea*. In this report, the phenolic acid composition and the linkage forms of the phenolic substances in the cell walls were also investigated. Furthermore, the activities phenylalanine ammonia-lyase (PAL), being a key enzyme in the biosynthesis of phenolic substances, and peroxidase, being involved in lignin biosynthesis, were investigated.

Materials and Methods

Callus culture

The carrot (*Daucus carota* L., cv. Kintoki) callus cells used in these experiments were originally induced from cambial explants of carrot root on Murashige-Skoog (MS) medium supplemented with 20 g l^{-1} sucrose, 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and 1% agar (pH 5.8). Formed callus cells were transferred to Schenk-Hildebrandt (SH) medium containing 20 g l^{-1} sucrose, 0.5 mg l^{-1} 2, 4-D, 0.1 mg l^{-1} kinetin and 1% agar (pH 5.8). The callus cells had been subcultured at three weeks intervals and maintained for four years in the dark at 25 ℃.

Culture of fungi

Botrytis cinerea was cultured on Potato-extract agar containing 2% sucrose, and grown at 25 ℃ in the dark. Conidia were harvested from eight- to ten-day-old cultures. Sterile water was added to the agar surface of cultures and gently stroked with a glass rod to dislodge the conidia. The spore suspension was filtered through three thickness layers of sterile cheese-cloth to remove mycelium fragments. The spore suspension was washed two times with sterilized water and the concentration adjusted to $1x10^6$ spores ml^{-1} (Uchiyama *et al.*, 1984).

HWE from conidia

For preparing HWE from conidia, the spore suspension ($1x10^6$ spores ml^{-1}) was autoclaved at 120 ℃ for 15 min, followed by centrifugation at 3000 *g* for 10 min. The supernatant was used as HWE of conidia.

Elicitation of carrot callus cells

Three groups of callus cell aggregates, each group consisting of 5-8 pieces (0.6-0.8 g wet weight), were transferred to Petri dishes (9 cm diameter) containing SH agar medium. Two groups of cell aggregates were treated on their upper surface with *B. cinerea* spore suspension ($1x10^4$-10^5) or 0.2 ml of conidia HWE with a sterile pipette. The remaining group was left untreated as a control. The Petri dishes were incubated at 25 ℃ in dark.

Estimation of free phenolics

Treated and non-treated callus cells were harvested at specific times of incubation, and lyophilized. Dried cells were ground in mortar and extracted with 50 ml of 80% methanol in water bath for 30 min. at 80 ℃, followed by filtration through glass fiber paper (Whatman GF/A). The filtrates

were concentrated to 5 ml by rotary evaporation, and extracted three times with ethyl acetate. The solution was evaporated in vacuo to dryness. The dried materials were dissolved in 5 ml of methanol and used for estimation of phenolic acid contents. Free phenolic substances were measured by the Folin-Ciocalteu method as gallic acid equivalent (Uchiyama *et al.*, 1983; Cahill and McComb, 1992). The 80% methanol insoluble residues (AIR) were used as starting materials for analysis of lignin and phenolic substances in cell walls.

Estimation of lignin contents

For lignin analysis, thioglycolic acid procedure was used (Hammerschnit, 1984; Bruce and West, 1989). AIR (50 mg) was placed in a test tube containing 0.5 ml of thioglycolic acid and 5 ml of 2 N hydrochloric acid. The tube was capped and heated at 95 ℃ for 4 h. After cooling, the solids were collected by centrifugation (12000 *g*, 15 min), and the supernatant was discarded. The solids were washed by re-suspension in 5 ml of water, followed by centrifugation (12000 *g*, 15 min). The washed solids were incubated in 5 ml of 0.5 N sodium hydroxide solution for 16-18 h to solubilize the lignin-thioglycolic acid (LTGA). After this extraction, the solids were removed by centrifugation (12000 *g*, 15 min). Each sodium hydroxide extracts was placed into a 15 ml conical centrifuge tube and 1 ml of concentrated hydrochloric acid was added. The acidified solution was held at 4 ℃ for 4 h to aid LTGA precipitation. The precipitated LTGA was collected by centrifugation (3000 *g*, 15 min), and washed twice with 0.1 N hydrochloric acid. The LTGA pellets were dissolved in 10 ml of 0.5 N sodium hydroxide, and the absorbance (A) at 280 nm was measured.

Preparation of cell wall materials

To remove proteins, the AIR was washed with phenol : acetic acid : water (2:1:1, W/V/V) for 24 h at room temperature, followed by centrifugation (9000 *g*, 20 min). The pellets were washed twice with water, followed by centrifugation. To remove amylose and amylopectin, the pellets were washed twice with 90% DMSO. The pellets were then washed several times with water, followed by centrifugation (10000 *g*, 15 min). The washed pellets were re-suspended in water, and dialyzed against water for one day. The suspended cell wall materials (CWM) were collected by centrifugation, and freeze-dried (Selvendra and Ryden, 1990). CWM was treated with 20 mM ammonium oxalate for 1 h at 70 ℃ (repeated two times), followed by centrifugation (10000 *g*, 15 min). The residues were further extracted to remove pectic substances with 30 mM oxalic acid for 3 h at 100 ℃, followed by centrifugation. The precipitate was freeze-dried, and the dried material designated as pectin-depleted CWM. The materials extracted from CWM with ammonium oxalate and oxalic acid were dialyzed with water, and freeze-dried, respectively (Kamisaka *et al.*, 1983).

Preparation of dioxane lignin

For preparing dioxane lignin (Asada and Matsumoto, 1972; Uchiyama *et al.*, 1983), CWM was extracted with a dioxane-hydrochloric acid solution for 12 h at 90-95 ℃, followed by filtration. The residue was washed with dioxane. The filtrate and washings were mixed, neutralized with sodium bicarbonate, and filtered. The filtrate was concentrated *in vacuo*, and then added acidic water (pH 2). The resulting precipitate was collected by centrifugation at 1600 *g* for 20 min. The precipitate was washed several times with water and dissolved in a small amount of dioxane. The dioxane solution was added to cold ethyl ether with stirring. The resulting precipitate was collected by centrifugation and dried *in vacuo*. The precipitate was designated as a dioxane lignin substance (DLS).

Analysis of phenolic composition of lignin

For analyzing the phenolic composition of lignin, alkaline nitrobenzene oxidation was carried out by the following procedure (Iiyama, 1990): specimens (10 mg of DLS, the total LTGA obtained from 50 mg AIR, and 10 mg of CWM) were degraded in a mixture containing 0.05 ml nitrobenzene and 3 ml of 2 N sodium hydroxide at 160 ℃ for 2 h in a block heater. For high temperature alkaline digestion was applied by following procedure (Iiyama, 1990): specimens (the total LTGA obtained from 50 mg of AIR, and 10 mg of CWM) were degraded in a mixture containing 3 ml of 2 N sodium hydroxide at 160 ℃ for 2 h in an aluminum block heater. For saponification at room temperature, specimens were treated with 0.5 N and/or 4 N sodium hydroxide. The released phenolic acids were extracted twice with ethyl ether, and the combined organic phase was dried with anhydrous sodium sulfate, followed by evaporation to dryness.

Phenolic materials were analyzed as their TMSi derivatives by GLC (3% OV-1, 2 mx3 mm) (Uchiyama *et al.*, 1983) and/or GC-MS (DB-1, wide bore capillary column, 30 mx0.54 mm), described previously (Yang and Uchiyama, 2000). Ethyl vanillin was used as internal standard (Iiyama, 1990).

PAL extraction and assay

Carrot callus cells (1 g fr. wt.) were grounded with a cold Potter-Elvehjem glass-teflon homogenizer in 2 ml of 0.1 M borate buffer (pH 8.8) containing 2-mercaptoethanol (1 mmole), and centrifuged at 12000 *g* for 15 min at 5 ℃. The supernatant was dialyzed against 0.01 M borate buffer (pH 8.8) for 20 h at 5 ℃. The dialyzed solution was used as a crude enzyme extract. Protein assay kit (Bio-Rad Lab.,) was used for estimation of proteins.

The enzyme activity was estimated in a mixture containing 100 mmole of L-phenylalanine, 50 μl of mercaptoethanol (1 mmole), 3 ml of borate buffer and 1 ml of crude enzyme solution. The enzyme reactions were incubated at 40 ℃ for 90 min, stopped with the addition of 6 N hydrochloric acid (0.5 ml), and centrifuged at 16000 *g* for 15 min at 5 ℃. The *t*- cinnamic acid formed in the reaction mixture was extracted from the supernatant with diethyl ether three times. The solvents were dehydrated with anhydrous sodium sulfate, followed by evaporation *in vacuo*. The residues were dissolved in ethanol (5 ml), and the optical density of formed *t* - cinnamic acid was measured at 273 nm (Uchiyama *et al.*, 1984).

Peroxidase extraction and assay

Carrot callus cells (1 g fr. wt.) were ground with a cold glass-teflon homogenizer in 5 ml of 0.1 M phosphate buffer (pH 6.3) and centrifuged at 12000 *g* for 15 min at 5 ℃. The supernatant was used as a soluble enzyme fraction. After washing with same buffer solution, wall proteins were extracted from the residues with 0.1 M phosphate buffer (pH 6.3) containing 1 M sodium chloride at 5 ℃ for 15 h with stirring, followed by centrifugation at 12000 *g* for 15 min. This supernatant was dialyzed against extraction buffer and used as ionically-bound enzyme. After the high salt extraction, the residues were washed again with extraction buffer, as before. The residues were then resuspended and stirred in enzyme mixture (8 ml) containing 0.6% cellulase (ONZUKA R-10, Yakult Co. Ltd.) and 0.6% pectinase (ICN Nutritional Biochemicals) in 0.1 M sodium acetate buffer (pH 5.0) for 15 h at 5 ℃. Insoluble matter was removed by centrifugation at 15000 *g* for 15 min, and the supernatant dialyzed against 0.01 M sodium acetate buffer. This dialyzed preparation was used as the covalently-bound enzyme fraction. Peroxidase activity was determined using guaiyacol and pyrogallol as substrate (Jaegher *et al.*, 1985; Cvikrova, *et al.*, 1994). Guaiacol-peroxidase activity was determined at 25 ℃ in 10 ml of phosphate buffer (pH 6.3) containing 0.3% guaiacol, 0.05% hydrogen peroxide and 25 μl enzyme. Enzyme activity was determined for three preparations and was defined as the guaiacol-dependent increase in absorbance at 470 nm per min and per mg protein. Pyrogallol-peroxidase activity was determined a reaction mixture containing 1 ml 0.5% pyrogallol solution, 0.1 ml 0.5% hydrogene peroxide and 1 ml enzyme solution in a total of 8 ml. Enzyme activity was determined for three preparations and was defined as the mg purprogallin formed (mg protein)$^{-1}$ min^{-1}. A protein assay Kit (Bio-Rad Lab.,) was used for estimation of protein.

Results

The response of carrot callus cells to elicitation

For assessing the response of carrot callus cells to inoculation with fungal pathogens, conidia of *Botrytis cinerea* were applied to the callus cells. One day after inoculation, little mycelial growth was observed, but after 48 h most surfaces of the carrot callus cells were lightly covered with mycelia. These results indicated that carrot callus cells had almost no ability to inhibit germination of conidia and mycelium growth of inoculated fungi. On the other hand, callus surfaces treated with conidia stained positive with phloroglucinol-HCl, evidence for lignin synthesis. The positive reaction was also observed after treating with heat-killed conidia or HWEs from conidia of *B. cinerea*. Color development was marked on cells that had been cultured for nine and ten days. Even if other reagents (Shiffs reagent, Mauls reagent) did not reveal evidence occurrence of lignification, the histochemical observation indicated that lignification was elicited in the carrot callus cells by treating with conidia, heat-killed conidia, or HWE from conidia of *B. cinerea*. Further experiments were carried out using HWE, because this was more inductive than other materials.

The concentration of free phenolic acids and lignins in carrot callus cells elicited with HWE from conidia of B. cinerea

The amounts of free phenolic substances scarcely increased in cell extracts of carrot callus (culture age-9 days) elicited with HWE of *B. cinerea* conidia (**Table 1**). On the other hand, the amounts of LTGA in cells elicited with HWE increased four-fold by 20 h after treatment, relative to the amount of LTGA at time 0 (**Table 2**). Carrot callus cells differing in the number of days they had been culture after subculture were elicited with HWE, and the changes in lignin content examined. **Fig. 1** shows the changes in the amounts of LTGA as a

Table 1 Changes in total content of free phenolic substances in carrot callus cells elicited with HWE from conidia of *B. cinerea*

Time after elicitation (h)	Free phenolic substances (μg gallic acid per g cell dry weight)	
	Non-elicited	Elicited
0	2.83 ± 0.22	-
4	-	2.83 ± 0.54
8	-	2.90 ± 0.10
12	2.93 ± 0.43	3.96 ± 0.38
24	2.77 ± 0.22	3.82 ± 1.48

Nine-day cultures were elicited with HWE of conidia. Phenolic substances were extracted with 80% of methanol, and measured by Folin-Ciocalteu method as gallic acid equivalent. Each value is the ± s.d. of three replicate experiments.
(- : not tested)

Table 2 Changes in the concentration of LTGA in carrot cells elicited with HWE from conidia of *B. cinerea*

Time after elicitation (h)	LTGA (A_{280})
0	0.41
8	0.65 ± 0.01
12	0.93 ± 0.02
16	1.21 ± 0.04
20	1.53 ± 0.01

Nine-day cultures elicited with HWE of conidia were harvested and lyophilized. Dried materials were homogenized in 80% methanol, followed by filtration. The insoluble cell materials (AIR) were lyophilized, and 50 mg AIR used for lignin determination. Each value is the mean ± s.d. of three replicate experiments.

function of the culture age of callus cells. The amount of LTGA in non-elicited cells was approximately constant at a low level, while the amount in treated cells increased gradually, becoming five times higher than non-elicited cells by day-twelve. Twelve-day-old callus cells showed the greatest response to elicitation.

The phenolic composition of the lignin in carrot callus cells elicited with HWE of B. cinerea.

The phenolic composition of LTGA prepared from 12-day-old elicited cells was investigated by simple alkaline hydrolysis and alkaline nitrobenzene oxidation. As shown in **Table 3**, only ρ-hydroxybenzoic acid (ρ-HBA) was eliminated from LTGA by alkaline hydrolysis, and the amounts were increased four-fold in the elicited cells rela-

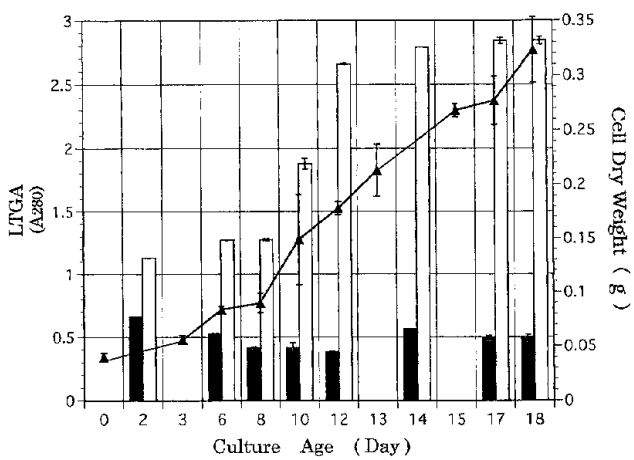

Fig. 1 Influence of culture age and elicitor treatment on lignin biosynthesis. Cultures of the indicated age were elicited (□) with HWE for 16 h, and the lignin was assayed from 50 mg AIR. Control cultures without elicitation (■) were harvested simultaneously and also assayed for lignin. The growth curve was measured as cell dry weight of non-elicited cells (-▲-). Each value is the mean ± s.d of three different experiments.

Table 3 Phenolic composition of LTGA

Hydrolysis procedure		Vanilline	Vanillic acid	ρ-Hydroxybennzoic acid
		(μg per mg AIR)		
Alkaline hydrolysis	Elicited	n.d.	tr.	0.659
	Non-elicited	n.d.	tr.	0.158
Alkaline nitrobenzene oxidation	Elicited	0.508	tr.	0.430
	Non-elicited	0.383	tr.	0.192

Twelve-day cultures were elicited with HWE from conidia for 16 h. LTGA prepared from AIR (50 mg) was subjected to alkaline nitrobenzene oxidation at 160 ℃. Phenolic materials were analyzed as their TMSi derivatives by GLC or GC/MS. Ethyl vanillin was used as internal standard. (tr.; traces)

Table 4 Phenolic composition in DLS

	ρ-Hydroxybenzalidehyde	Vanilline	Syring acid	Vanillic acid	ρ-Hydroxybennzoic acid
		(μg per mg dioxane lignin)			
Elicited	9.6	103.5	8.3	16.6	160.4
Non-elicited	13.4	91.2	6.5	14.7	87.4

DLS was extracted from CWM prepared from twelve-day cultures. DLS (10 mg) was subjected to alkaline nitrobenzene oxidation at 160 ℃. Phenolic materials were analyzed as their TMSi derivatives by GLC or GC/MS. Ethyl vanillin was used as internal standard.

Table 5 Amount and phenolic composition of lignin in carrot callus cells different in culture age

Culture age (Day)		LTGA	Vanillin	ρ-Hydroxybennzoic acid
		(A_{280})	(μg per mg AIR)	
9	Elicited	1.045	0.676	0.619
	Non-elicited	0.516	0.486	0.334
12	Elicited	1.291	0.952	0.926
	Non-elicited	0.425	0.457	0.193
16	Elicited	0.918	0.834	1.058
	Non-elicited	0.529	0.556	0.183

Cultures of the indicated age were elicited with HWE for 16 h. Amounts of lignin were estimated as LTGA. LTGA prepared from AIR (50 mg) was subjected to alkaline nitrobenzene oxidation at 160 ℃. Phenolic materials were analyzed as their TMSi derivatives by GLC or GC/MS. Ethyl vanillin was used as internal standard.

tive to non-elicited cells. On the other hand, the phenolic substances liberated by alkaline nitrobenzene oxidation were ρ-HBA and vanillin (Van) (**Table 3**). The amount of ρ-HBA increased in the elicited cells, but there were no marked differences in the amount of Van between elicited and non-elicited cells.

DLS was prepared from both elicited and non-elicited cells (culture age-twelve days), and the phenolic composition was examined by alkaline nitrobenzene oxidation. The major phenolic substances liberated from the DLS were ρ-HBA and Van, and the minor components were vanillic acid, ρ-hydroxybenzaldehyde, and syringaldehyde. Only the amount of ρ-HBA markedly increased in the elicited cells (**Table 4**).

Carrot callus cells differing in culture age were elicited with HWE of conidia, and the changes in the concentration and phenolic composition of the lignin (LTGA) were investigated. As shown in **Table 5**, the concentration of both ρ-HBA and Van in alkaline nitrobenzene oxidation products of LTGA from elicited cells increased with culture age. The amount of ρ-HBA responded more dramatically to elicitation than that of Van, and showing five-fold increase when cells of 12- and 16-days culture age were elicited.

The concentration and composition of phenolic acids in the CWM from cells elicited with HWE of conidia were examined by successive extraction with 0.5 N and 4 N sodium hydroxide, because wall bound phenolic acids were released by sequential alkaline hydrolysis of wall material under vigorous condition (Hartley and Morrison, 1991), and followed by alkaline nitrobenzene oxidation. ρ-HBA was the only phenolic substance liberated by hydrolysis with 0.5 and 4 N sodium hydroxide; the amount released from CWM increased two- and four-fold, respectively, after elicitation with HWE of conidia (**Table 6**). The phenolic acid composition of the residual CWM, after successive alkaline hydrolysis, was examined by alkaline nitrobenzene oxidation. ρ-Hydryoxybenzaldehyde, ρ-HBA and Van were detected and their amounts were nearly equal, whether from elicited or non-elicited cells.

The concentration and composition of phenolics in the CWM prior to successive alkaline hydrolysis were examined with alkaline nitrobenzene oxida-

Table 6 Phenolic composition in CWM

Hydrolysis procedure		ρ-Hydroxybenzalidehyde	Vanilline	ρ-Hydroxybennzoic acid
		(μg per mg CWM)		
0.5 N-alkaline hydrolysis	Elicited	n.d.	n.d.	0.84
	Non-elicited	n.d.	n.d.	0.44
4 N-alkaline hydrolysis	Elicited	n.d.	n.d.	2.50
	Non-elicited	n.d.	n.d.	0.58
Nitro-benzene oxidation	Elicited	0.40	2.71	0.33
	Non-elicited	0.36	2.21	0.32

Phenolic materials in the CWM depleted pectic substances (300 mg) were investigated by successive extraction with 0.5 N and 4 N sodium hydroxide. The final residue was subjected to alkaline nitrobenzene oxidation at 160 ℃. Phenolic materials were analyzed as their TMSi derivatives by GLC or GC/MS. Ethyl vanillin was used as internal standard.

Table 7 Phenolic substances liberated by alkaline hydrolysis of CWM methylated with diazaomethane in methanol

		ρ-Methoxybennzoic acid	ρ-Hydroxybennzoic acid
		(Area %)	
Elicited	0.5 N-NaOH	27.86	6.15
	4 N-NaOH	25.48	8.32
Non-elicited	0.5 N-NaOH	9.62	0.66
	4 N-NaOH	8.83	1.55

Pectin-depleted CWMs (100 mg) were methylated with diazomethane in methanol. Phenolic materials in methylated CWM were liberated by alkaline hydrolysis. Phenolic materials were analyzed as their TMSi derivatives by GC/MS and estimated as area % of the corresponding peak.

tion. Phenolic acids present were ρ-hydroxybenzaldehyde, ρ-HBA and Van. The amount of ρ-HBA markedly increased in HWE-elicited cells (data not shown).

ρ-Hydroxybenzoic acid in the cell wall of carrot callus

As described above, the major phenolic acids detected in the lignin of HWE-elicited carrot callus cells were ρ-HBA and Van, and ρ-HBA was particularly elevated. ρ-HBA was easily liberated from CWM by alkaline hydrolysis. These results indicated that the ρ-HBA that increased in the HWE-elicited cells was ester-linked with lignin-like precursors or wall polysaccharides. Therefore, pectin-depleted CWM was directly methylated with diazomethane in methanol, and alkaline hydrolysable phenolic acids from the methylated CWM were investigated. As shown in **Table 7**, the concentration of both ρ-HBA and ρ-methoxy benzoic acid increased in the CWM from cells elicited with HWE. The results indicated that the increased ρ-HBA was ester-linked via its carboxylic groups to hydroxyl groups of lignin precursor or wall polysaccharides and/or via its hydroxyl groups to carboxylic groups in lignin precursors. In addition, the increase in ρ-methoxybenzoic acid also indicated that the carboxylic group of ρ-methoxybenzoic acid was ester-linked to hydroxyl groups in lignin precursor or wall polysaccharides.

PAL and peroxidase activity in carrot callus cells elicited with HWE of conidia

Changes in PAL activity in carrot callus cells elicited with HWE of conidia were investigated. PAL activity increased and attained its maximum at 16 h after eliciting with HWE (**Fig. 2**).

Callus cells differing in culture age were elicited

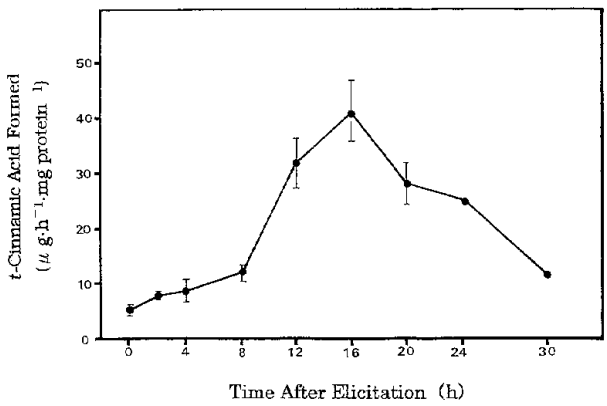

Fig. 2 Time course of phenylalanine ammonia-lyase activity in carrot callus cells elicited with HWE. Callus cells cultured for twelve day were used. Elicitor was applied at time 0. Each value is the mean ± s.d of three replicate experiments.

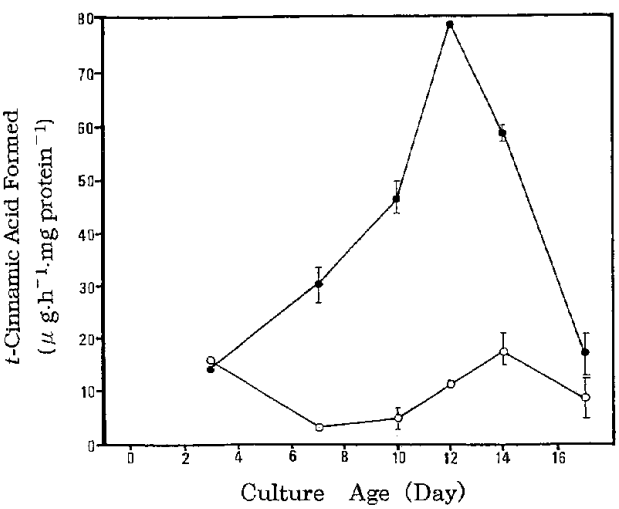

Fig. 3 Influence of culture age and elicitor treatment on PAL activity. Cultures of indicated age were elicited (-●-) with HWE for 16 h, and the PAL activity was assayed from 1 g fresh weight. Non-elicited controls (-○-) were harvested simultaneously and also assayed for PAL activity. Each value is the mean ± s.d of three different experiments.

with HWE, and the changes in PAL activity examined. PAL activities in the non-elicited callus cells was maintained at a low level, but the activity in elicited callus cells increased with culture age, showing five-fold induction in twelve-day-old cells relative to non-elicited cells, followed by a rapid decline within the next four days back to the control level (**Fig. 3**).

Changes in peroxidase activity in callus cells (culture age twelve-day) elicited with HWE of conidia were also investigated using pyrogallol and guaiyacol as substrate. Neither soluble nor ionically-bound peroxidase activity was affected by eliciting with HWE. However, covalently-bound peroxidase activity was high at first (0 h), but shortly afterward marked decrease of the activity occurred (**Fig. 4**).

Discussion

Carrot callus cells were elicited with HWE from conidia of *B. cinerea* and formed lignin or lignin-like materials. That lignification was induced in the elicited cells was supported by the increases observed in LTGA and PAL activity. But peroxidase activity, believed to accompany for lignification, did not increased after elicitation with HWE of conidia.

The phenolic acid composition of LTGA was compared between HWE-elicited and non-elicited cells using mild alkaline hydrolysis and alkaline nitrobenzene oxidation. Only *p*-HBA was liberated by alkaline hydrolysis, and the amount rose to four-fold in the HWE-elicited cells. On the other hand, the products of alkaline nitrobenzene oxidation were *p*-HBA, which increased two-fold in elicited cells, and Van, which showed no change. To confirm the phenolic acid composition of LTGA, DLS was prepared from elicited and non-elicited cells, and the composition examined by means of alkaline nitrobenzene oxidation. *p*-HBA and Van occurred more abundantly in DLS, and the amount of *p*-HBA increased two-fold in the elicited cells. These results indicated that *p*-HBA was significantly more abundant in both LTGA and DLS extracted from HWE-treated cells.

Asada and Matsumoto (1972) examined induced lignin in Japanese radish inoculated with *Peronospora parasitica* to clarify the differences between lignin from healthy and diseased tissues. They reported that the lignin formed in diseased tissues was of the guaiacyl type, but in healthy tissues was of the syringyl type. From the detection of Van in the lignin (LTGA and DLS) of carrot callus cells, irrespective of elicitation, it was surmised that guaiacyl type lignin was present. Although no qualitative differences were found in phenolic acid composition of the lignin from elicited versus non-elicited carrot callus cells, the amount of *p*-HBA increased after elicitation. The phenolic acids *p*-HBA and Van were also found in LTGA from HWE-elicited cells differing in subculture age, but only the amount of *p*-HBA increased. In addition, *p*-HBA was liberated from pectin-depleted CWM from elicited and non-elicited cells by successive alkaline hydrolysis (0.5 N and 4 N sodium hydroxide), and the amount increased in the elicited-cells. From these results, it was deduced that the *p*-HBA

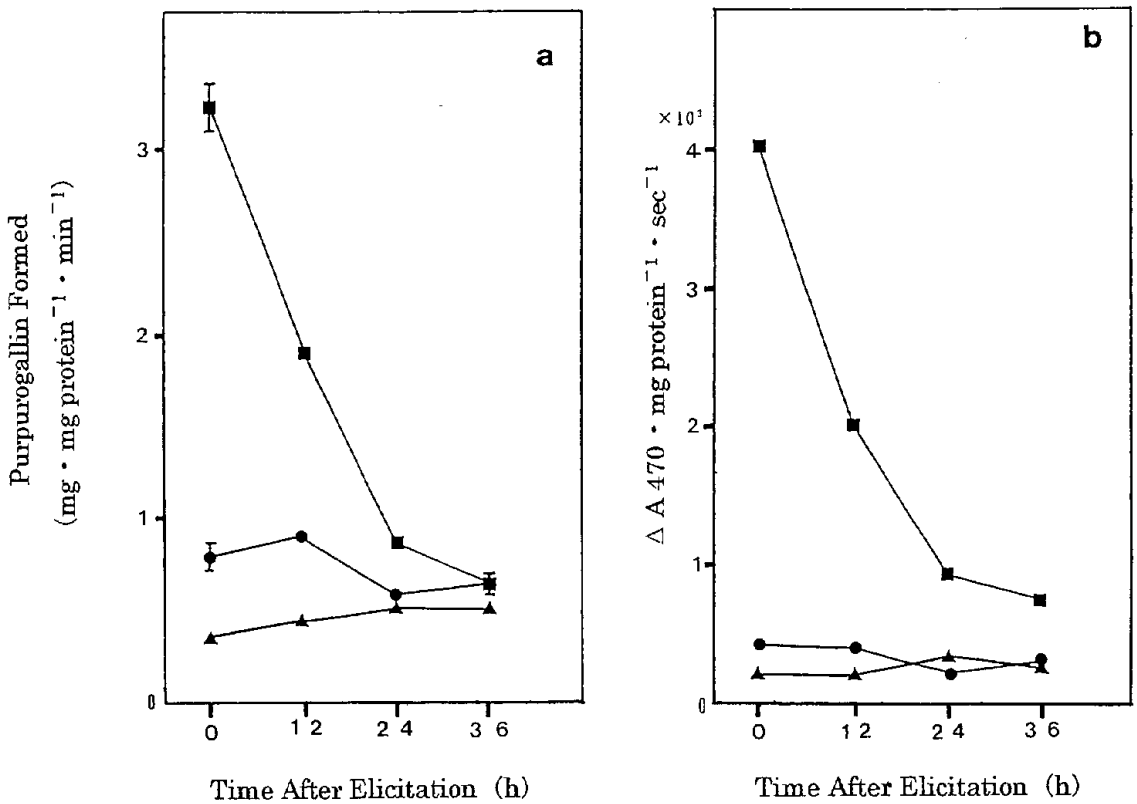

Fig. 4 Changes of soluble (●), ionically-bond (▲), and covalently-bond (■) peroxidase activity in the carrot callus cells elicited with HWE. Peroxidase activity was determined using pyrogallol (A) and guaiyacol (B) as substrate.
Callus cells cultured for twelve day were used. Elicitor was applied at time 0. Each value is the mean ± s.d of three different experiments. A: Enzyme activity was defined as the mg purprogallin formed (mg protein)$^{-1}$ min^{-1}. B: Enzyme activity was defined as the guaiacol-dependent increase in absorbance at 470 nm per min and per mg protein.

that increased in the elicited cells was ester-bonded to cell wall materials such as lignin precursors or wall polysaccharide. Consequently, pectin-depleted cell walls were directly methylated with diazomethane in methanol, and alkaline hydrolysable phenolic acids were investigated. In this experiment, both *p*-methoxybenzoic acid and *p*-HBA were defined, and their amounts increased in elicited cells three-fold and ten-fold, respectively. These results indicated that the increased *p*-HBA was ester-bonded to lignin precursors or wall polysaccharides with both its hydroxyl and carboxyl groups.

Plant wall polysaccharides contain a small number of ester-linked phenolic acid side chains. For example, Ishii and Hiroi (1990) reported the structure of a feruloylated xyloglucan disaccharide in bamboo shoot cell wall. Yang *et al.* (2000 a, b) reported that the cell wall polysaccharides in *Mentha* suspension-cultured cells formed ester bonds with both ferulic and caffeic acid. These ester-bonded side chains can undergo dimerization though wall-bound peroxidase-mediated oxidative coupling to produce dehydrodimers, and these dimers contribute to cessation of cell elongation, and inhibition of wall degrading enzymes (Yang and Uchiyama, 2001). But the signals which induce oxidative coupling and cross-linkage of cell wall polysaccharides are as yet unclear.

Dehydrodimers of *p*-HBA, such as ferulic acid and caffeic acid, cross-linked among wall polysaccharides, could not be detected in the elicited carrot callus cells. Furthermore, peroxidase activity did not increase after elicitation, even through enhancement of peroxidase activity has been known to participate in lignin biosynthesis and oxidative coupling of phenolic acids (Fry, 1983; Cvikrova, *et al.*, 1994; Yang and Uchiyama, 2000). This lack of enhancement of peroxidase activity in elicited cells may be evidence that dehydrodimers of phenolic acids were not formed. The causes of inactivation of peroxidase in the HWE-treated carrot callus cells are still unclear, but Messner *et al.* (1994) reported that the extracellular peroxidases of suspension cultures of spruce (*Picea abies*) become inactivated when the cell suspension is elicited with a cell wall preparation of the spruce pathogenic fungus (*Rhizosphaera kalkhoffii*). Van Lelyveld *et al.* (1978)

reported that *Phytophthora cinnamomi* induced peroxidase inhibitors in avocado leaves. Inactivation of peroxidase in the HWE-treated carrot callus cells might be caused by unknown inhibitors in fungal elicitors (HWE).

There are many studies of the induced lignification of cultured cells in response to inoculation with fungal pathogens, but these previous studies have failed to distinguish induced lignin from ester-bonded phenolic substances known to form cross-linkage in cell wall polysaccharides. Regardless of what the material is, lignin or lignin-like materials in cells adjacent to injuries were detected with phloroglucinol-HCl reagent. But ρ-HBA did not react with this reagent. Friend (1981, 1982) has suggested that the lignin-like material produced in potato tuber in response to *P. infestans* consists partly of cell wall bound ester of ρ-coumaric and ferulic acid and cross-linked wall bound phenolic acids (Hammerschmidt, 1984).

Elicited lignification in cultured carrot cells may indicates an increase of ester-bonded phenolic acids (ρ-HBA) in lignin precursor or wall polysaccharides rather than de novo synthesis of lignin. The increase in ester-bonded ρ-HBA in HWE-treated cells strongly suggests this possibility.

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