イチゴ青枯病に関する研究 (2)

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Studies on Bacterial Wilt of Strawberry Plants Caused by *Pseudomonas solanacearum* 2. β-D-Glucogallin, the Antibacterial Substance Detected in the Tissues of Strawberry Plants

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川口邦男* ・ 太田啓一** ・ 後藤正夫* ：イチゴ青枯病に関する研究
2．イチゴ組織に見出された抗細菌物質 β-D-glucogallin について

Abstract

An antibacterial substance inducing growth inhibition of *Pseudomonas solanacearum* on agar plates was isolated from strawberry leaves by Sephadex G-25 and Avicel column chromatography. The substance was identified as β-D-glucogallin (β-1-O-galloyl-D-glucose) by paper chromatography, UV absorption spectrum patterns, and acid hydrolysis for determination of the components. The β-D-glucogallin contents of strawberry plants positively correlated with the antibacterial activity in terms of inhibition zone. In assays during the period of one month after inoculation, no detectable change was observed in the content of β-D-glucogallin as well as the antibacterial activity. Extracts taken from younger leaves inhibited the growth of *Ps. solanacearum* most effectively, those from petioles being next. Extracts from stems and roots showed a lower inhibitory activity compared with the above mentioned. Antibacterial activity of the tissues varied depending on the plant age and growing seasons. The crude extracts from strawberry leaves inhibited not only growth of *Ps. solanacearum* but also that of some other bacteria such as *Ps. meliae*, *Ps. syringae* pv. *pisi*, *Ps. syringae* pv. *lachrymans*, *Pseudomonas* sp. from *Ficus erecta*, *Ps. andropogonis* and *Xanthomonas campestris* pv. *oryzae*. No growth inhibition was observed against the other pathovars of *X. campestris*, erwiniae and corynebacteria. However, production of extracellular polysaccharides was significantly depressed in most of the pathovars of *X. campestris*.

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Introduction

Bacterial wilt of strawberry plants caused by *Pseudomonas solanacearum* (E. F. Smith) E. F. Smith is found on younger seedlings in nurseries, but rarely on mature plants growing in fields. Inoculation tests also revealed that mature plants have strong resistance to the disease. The bacterium invaded a restricted number of tracheal elements even in the younger seedlings, and attacked instead the parenchymatous tissues forming large lysigenous cavities with bacterial cells. Although the mature plants exhibit such strong resistance, they often carry the pathogenic bacteria in the stem tissues, the ratio of carrier plants being dependent on the kind of cultivars. During the investigations on the

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nature of resistance, we found that the growth of *Ps. solanacearum* was strongly inhibited when pieces of leaf petiole, stem or root tissues of strawberry plants were placed on nutrient agar plates seeded with the bacterium. The substances inhibiting bacterial growth seemed to be responsible for the resistance of strawberry plants. Therefore, studies were conducted to identify and characterize the antibacterial substances contained in healthy plant tissues. Assays were also made to analyse the distribution of these substances in various organs of strawberry plants along with the seasonal fluctuation of their concentrations.

Materials and Methods

Bacteria. A strawberry isolate, SB3-9, of *Ps. solanacearum* was used throughout the study as the indicator strain for screening growth inhibitory substances. The culture was maintained on potato-sucrose-agar slants frozen at −30 C and in sterilized tap water at room temperature. Other bacteria used for the activity spectra of growth inhibitory substances were provided by the culture collection of phytopathogenic bacteria at the Laboratory of Plant Pathology, Shizuoka University.

Media. Peptone-sucrose medium adjusted to pH 6.8 was used in the study unless otherwise mentioned. It contained 1.0 % peptone and 1.0 % sucrose with or without agar.

Plants. Two cultivars of strawberry (*Fragaria chiloensis* Dach. var. *ananasa* Bailey), Hoko Wase and Hotta Wander, were used. The seedlings were grown in flat boxes (40×50×10 cm in size and 8 plants per box) which were placed in a greenhouse.

Inoculation method. The roots of strawberry plants were cut with a sharp knife through soil and the bacterial suspension was poured on the cut surface of the roots. The inoculated plants were kept in a greenhouse for observation of disease development.

Separation and purification of antibacterial substances. Thirty grams of fresh leaves of strawberry plants were cut into pieces and heated at 80 C for 2 hr in 75 ml of deionized water. The supernatant was filtered through filter paper. Separation and purification of antibacterial substances from the filtrate were performed by Sephadex G-25 and Avicel column chromatography. Sephadex G-25 was suspended in distilled water and packed into the column (2×15 cm). The dried crude extracts were dissolved in a small volume of distilled water, placed on top of the column, and eluted with distilled water. The eluate was collected in 10 ml-fractions. Avicel was suspended in acetone and packed into the column (2×15 cm). The column was equilibrated with 2 % acetic acid or *n*-butanol saturated with water and eluted with them. The eluate was collected in 5 ml-fractions. The fractions obtained from the column chromatography were monitored by paper partition chromatography (PPC). Spots were detected on one dimensional PPC with UV-light and potassium ferricyanide-ferric ammonium sulfate.

Bioassay. A separated F-type colony was taken from nutrient agar plates, and grown in peptone-sucrose broth at 28 C for 20 hr on a reciprocal shaker with 7 cm amplitude at a frequency of 100 strokes per min. 0.1 ml of the bacterial growth was mixed with 5 ml of melted peptone-sucrose agar and spread on a solidified layer of the same medium (10 ml) in a Petri dish.

Five paper disks (Toyo Roshi, 8 mm in diam., 2 mm thick) were dipped in each sample and dried in vacuum. They were put on the surface of agar plates seeded with

Ps. solanacearum and incubated at 28 C for 18 hr. Diameters of inhibition zones were recorded and expressed by the mean value of five disks.

Identification of the antibacterial substances. Identification of the antibacterial substances was carried out by comparison of Rf values of PPC, UV absorption-spectrum patterns and hydrolysis with mineral acid. One dimensional PPC was carried out on Toyo Roshi No. 50 paper with the following solvents: (A) 2% acetic acid; (B) 6% acetic acid; (C) *n*-butanol-acetic acid-water (4 : 1 : 2); (D) *sec*-butanol-acetic acid-water (14 : 1 : 5). UV-spectra were measured with a Hitachi 200-20 spectrophotometer. The samples were dissolved in 1.5% H_2SO_4 and kept at 100 C for 1 hr for the hydrolysis. After being cooled, the hydrolysate was extracted with ethylacetate. The ethylacetate and aqueous layers were separately developed on Toyo Roshi No. 50 paper. The spots were detected by spraying either anilinehydrogen-phthalate reagent or potassium ferricyanide-ferric ammonium sulfate reagent.

Quantitative assay of β-D-glucogallin in crude extracts. Methods described by Nakagawa and Torii[5] were applied. The crude extracts (20 μl) were spotted on Toyo Roshi No. 50 paper (20×20 cm) and then developed two dimensionally with 2% acetic acid and *n*-butanol-acetic acid-water (4 : 1 : 2). The dried paper chromatograms were sprayed with a minimum volume of diluted diazotized sulfanilic acid for detection of β-D-glucogallin. The diazotized sulfanilic acid was prepared by mixing the same volumes of 0.5% sulfanilic acid solution in 1.75% HCl and 5% sodium nitrate solution. This reagent was used after being diluted to 1/5 concentration with distilled water. The mixture of the reagent with 2.5 volumes of 2% sodium acetate buffer was used for the assay due to color reaction. The detected spots were cut and β-D-glucogallin was eluted with 4.5 ml of hot water (80 C). 0.18 ml of diazosulfanilic acid solution buffered with sodium acetate was added to the eluates and made up to 5 ml with distilled water. The coloured eluates were placed in a water bath at 35 C for 30 min. When the eluates cooled to room temperature, the absorbance at 420 nm was measured with a spectrophotometer. The amount of β-D-glucogallin was determined using the standard calibration curve of gallic acid.

Results

The separation procedures from extracts of strawberry leaves are shown in Fig. 1. Fraction III-1-2 was finally obtained as an active component from the column chromatography. This fraction gave positive color reaction on paper chromatography with the potassium ferricyanide-ferric ammonium sulfate, but not with HCl-vanillin and UV-light. Fig. 2 shows the UV absorption spectrum patterns of the fraction and gallic acid recorded by a double beam spectrophotometer. The $UV_{\lambda max}$ values of these substances in methanol were detected at 272 nm. From the hydrolysate of the fraction III-1-2 with 1.5% H_2SO_4, gallic acid and D-glucose were identified by paper chromatography. In the Rf values on paper chromatography given in Table 1, the fraction III-1-2 was identified as β-D-glucogallin (β-1-O-galloyl-D-glucose). The results indicated that the antibacterial substance in the extracts was β-D-glucogallin. The leaves and petioles of strawberry plants 3, 7, and 30 days after inoculation were assayed for β-D-glucogallin content and antibacterial activity in terms of growth inhibition zone with non-inoculated plants as

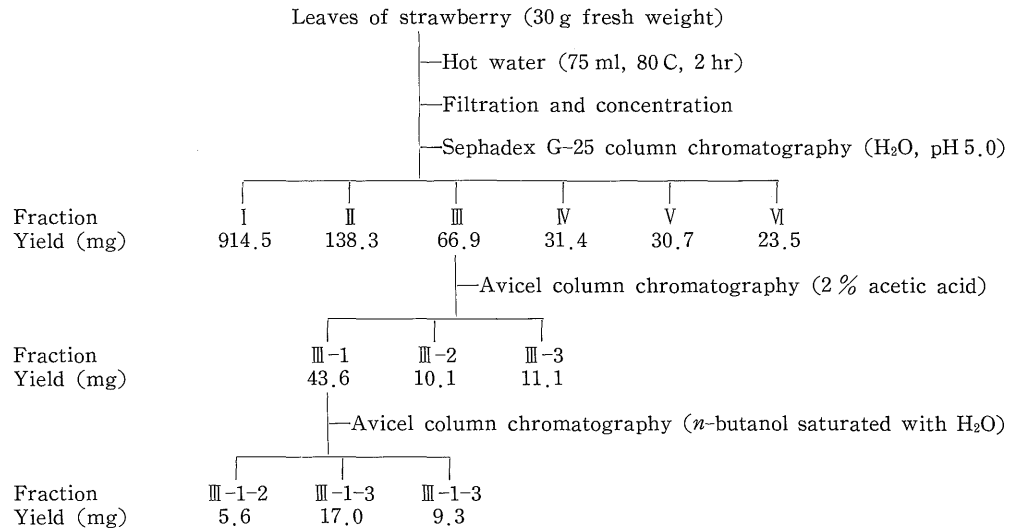

Fig. 1. Separation procedure of antibacterial substances from extract of strawberry leaves

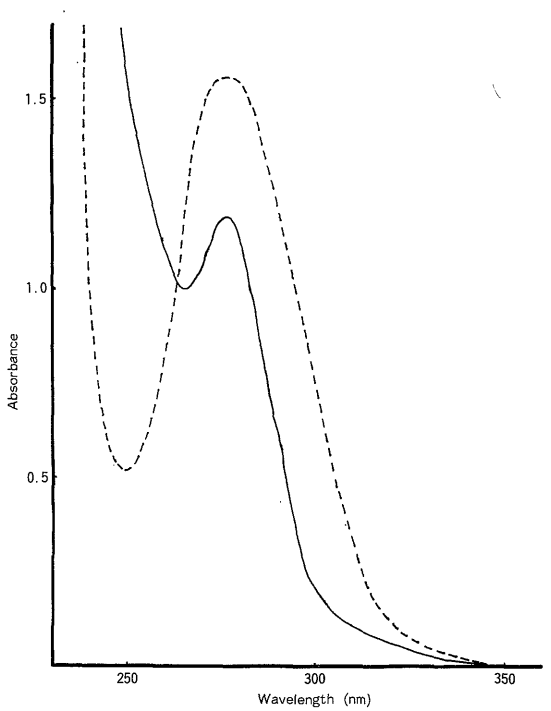

Fig. 2. UV absorption spectra of fraction III-1-2 and gallic acid in MeOH
——: Fraction III-1-2, ……: Gallic acid

Table 1. Comparison of Rf values on PPC

Solvent	Rf value	
	β-D-glucogallin[a)]	Fraction III-1-2
BAW (1)[b)]	0.30	0.27
BAW (2)[c)]	0.35	0.35
6 % AcOH	0.75	0.75
2 % AcOH	0.76	0.74

a) The Rf values incited from the reference[4)]
b) *sec*-BuOH : AcOH : H_2O (14 : 1 : 5)
c) *n*-BuOH : AcOH : H_2O (4 : 1 : 2)

the control. No detectable differences were observed in the content of β-D-glucogallin and/or the antibacterial activity between the inoculated and uninoculated plants (Table 2). The extracts from younger leaves showed the greatest activity among those from various organs of strawberry plants. (Table 3). The extracts from petioles became next. The extracts from roots and stems were less active. These results suggest that β-D-glucogallin is primarily produced in leaves and transferred to the other parts. The antibacterial activity was generally low in younger seedlings, but increased gradually as the plants grew, reaching the maximum value at the time of flowering and fruit-setting. The antibacterial activity decreased, however,

Table 2. Change of antibacterial activity and β-D-glucogallin content in strawberry plants inoculated with *Ps. solanacearum*

Cultivar	Organ	Antibacterial activity and β-D-glucogallin content	Days after inoculation			
			0	3	7	30
Hoko Wase	Petiole	Inhibition zone (diam. in mm)	15.2	15.0	14.1	14.0
		β-D-glucogallin (μg/g fresh wt.)	820	790	740	690
	Leaf	Inhibition zone (diam. in mm)	14.4	14.0	14.5	15.1
		β-D-glucogallin (μg/g fresh wt.)	750	715	790	825

Table 3. Antibacterial activity and β-D-glucogallin content in the plant organs of strawberry

Organ		Inhibition zone (diam. in mm)	β-D-glucogallin (μg/g fresh weight)
Leaf	young	17.0	980
	matured	14.0	700
	aged	11.0	450
Petiole	young	15.5	860
	matured	12.0	550
	aged	12.0	550
Stem		0	250
Root		0	275

with the delay in the growth of the plants in autumn (Fig. 3).

Growth inhibition activities of the strawberry leaf extracts and gallic acid solution were tested on various plant pathogenic bacteria (Table 4). Both samples inhibited the growth of *Ps. solanacearum*, *Ps. meliae* and *Pseudomonas* sp. from *Ficus erecta*. Activity of gallic acid was lower than that of leaf extracts. *Ps. andropogonis* (strelitzia strain), *Ps. syringae* pv. *pisi* and *Ps. syringae* pv. *lachrymans* were inhibited only by the leaf-extracts. The other pseudomonads were resistant to these substances.

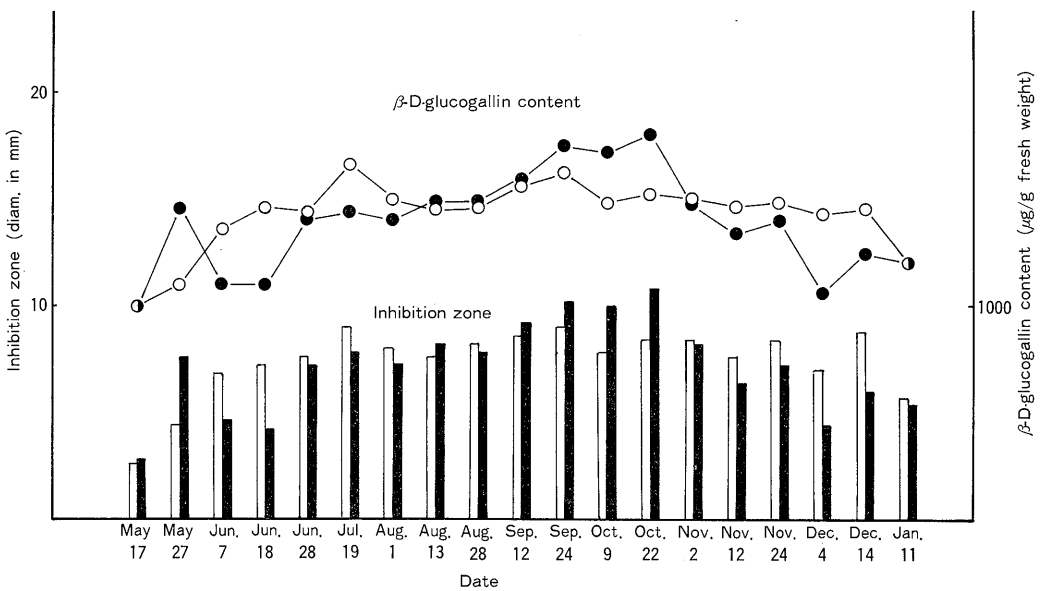

Fig. 3. Seasonal change of antibacterial activity and β-D-glucogallin content in strawberry leaves
○——○, □: Hoko Wase, ●——●, ■: Hotta Wander

Table 4. Sensitivity of plant pathogenic bacteria to extract of strawberry leaves and gallic acid solution

Bacteria	Isolate no.	Diameter of inhibition zone (mm)	
		Crude extract (20 mg f. wt./disk)	Gallic acid (0.5 mg/disk)
Pseudomonas solanacearum	S B3-9	16.0	14.5
	A1	17.5	14.0
	S1-1	18.0	14.0
	B4-4	16.0	14.5
	E1-1	19.5	15.0
	T16	22.0	15.0
Pseudomonas andropogonis	a	16.5	0
	d	16.5	0
	1	0	0
Pseudomonas sp. from *Ficus erecta*	L5	19.0	10.0
	L8	20.0	9.0
Pseudomonas sp. from *Amorphophalus konjac*	C	0	0
	f	0	0
Pseudomonas sp. from *Photinia glabra*	h	0	0
	i	0	0
Pseudomonas syringae			
pv. *pisi*	2	15.0	0
	3	15.5	0
pv. *theae*	1	0	0
	2	0	0
pv. tabaci	6820	0	0
	6707	0	0
pv. *maculicola*	1	0	0
pv. *syringae*	a	0	0
	b	0	0
pv. *phaseolicola*	1	0	0
pv. *lachrymans*	a	15.0	0
pv. *mori*	1	0	0
pv. *japonica*	A1	0	0
	P1	0	0
pv. *eriobotryae*	28	0	0
	N3	0	0
pv. *striafaciens*	4	0	0
Pseudomonas meliae	1	13.5	0
	7	0	0
Pseudomonas cissicola	C W5	0	0
Xanthomonas campestris pv. *vitians*	1	22.0*	10.0
pv. *begoniae*	1	20.0*	0
pv. *pruni*	1	20.0*	0
	5	21.5*	0
pv. *oryzae*	7221-3	17.5	17.0
	7228-3	18.0	16.0

Table 4. (continued)

Bacteria	Isolate no.	Diameter of inhibition zone (mm)	
		Crude extract (20 mg f. wt./disk)	Gallic acid (0.5 mg/disk)
pv. *citri*	U3	22.0*	10.0
	U4	21.0*	10.0
	U47	23.0*	10.0
pv. *pisi*	1	18.5*	0
pv. *campestris*	A	22.0*	0
	G	21.0*	0
pv. *nigromaculans*	XN1	21.0*	0
	XN4	21.5*	0
pv. *physalidicola*	1	20.0*	0
pv. *tardicrescens*	I2	16.5*	0
Erwinia carotovora subsp. *carotovora*	EC-1	0	0
Erwinia milletiae	1	0	0
Erwinia herbicola	A	0	0
	B	0	0
Corynebacterium michiganense pv. *michiganense*	1	0	0
Corynebacterium flaccumfaciens pv. *oortii*	RQ1	0	0
	M	0	0

* Inhibition of production of extra-cellular polysaccharides.

Among the various xanthomonads tested, the leaf-extracts and gallic acid inhibited only the growth of *X. campestris* pv. *oryzae*. However, the colonial appearance around paper disks suggested that β-D-glucogallin inhibits the slime production in many xanthomonads.

Discussion

The antibacterial substance in strawberry plants was identified as β-D-glucogallin. This substance has been found in Chinese rhubarb[4], *Terminaria chebula*[4] and astringent persimmon[6], but its antibacterial property has not been reported. The antibacterial activity was greater in β-D-glucogallin than gallic acid which is the aglycon form of the former. The reason for this phenomenon remains to be elucidated.

Bacterial cells in the inhibition zone on plates showed long chains. Bacterial growth occurred when a piece of agar block was taken from the inhibition zone and inoculated to liquid medium. These facts indicate that β-D-glucogallin acts as a bacteriostatic agent for *Ps. solanacearum*.

Among the numerous antibiotic substances detected in various kinds of plants[1,8], some are either phytoalexins produced after microbial infection or compounds previously present in healthy plants. Because the β-D-glucogallin content of strawberry plants and/or antibacterial activity showed no significant fluctuation after inoculation of *Ps. solanacearum*, it is suggested that the substance is natural component of healthy strawberry plants, but not a phytoalexin.

Ohigashi and Mitsui[7] reported that the methanol extracts from young leaves of about 40% of plant species inhibited the growth of *Bacillus subtilis*. From these results, they suggested that antibacterial substances are commonly contained in the younger plant

organs or tissues. Our results were also in agreement with their findings.

Goto *et al.*[3] reported that one of the characteristics of bacterial wilt of strawberry was the plugging of xylem tissues in a very restricted number of tracheal elements. However, large lysigenous cavities were often found in parenchymatous tissues, but rarely in the xylem. These facts suggest that β-D-glucogallin synthesized in the leaf lamina is transported to the other organs through vascular systems so as to increase the concentration in the tissues and to inhibit effectively growth of *Ps. solanacearum* in the xylem.

The crude extracts from strawberry leaves induced growth inhibition not only in *Ps. solanacearum*, but also in some members of *Pseudomonas* and *Xanthomonas*. In the xanthomonads, the production of extra-cellular polysaccharides was depressed although growth itself was not inhibited. Studies on the selective inhibition of slime production are underway.

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和 文 摘 要

イチゴ青枯病に関する研究 2. イチゴ組織に見出された抗細菌物質 β-D-glucogallin について

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Pseudomonas solanacearum をまいた寒天平板上にイチゴの植物組織片を置くと，増殖阻止円を形成した。この抗菌物質をセファデックス G25 およびアビセルカラムクロマトグラフィーによって単離した。この物質はペーパークロマトグラフィー，紫外線吸収スペクトルおよび加水分解産物の種類などから β-D-glucogallin と決定された。この物質の植物体内含量と抗菌活性の間には，正の相関関係が見られたが，その活性は病菌接種後においても変化しなかった。イチゴの器官別抗菌活性は若い上位葉ほど高く，葉柄がこれに次ぎ，茎および根では低かった。また秋の気温低下に伴ってイチゴ植物体の生長率が下がると，抗菌活性も低下した。葉の粗抽出物を用いて抗菌スペクトルを調べた結果，*Ps. solanacearum* の外，*Ps. meliae*, *Ps. syringae* pv. *pisi*, *Ps. syringae* pv. *lachrymans*, *Xanthomonas campestris* pv. *oryzae* 外 2 種の細菌に増殖阻止円を形成した。他の *Xanthomonas* 属細菌に対しては，増殖阻害は起こさなかったが，細胞外多糖質の生産を阻害した。*Corynebacterium* および *Erwinia* 両属の細菌に対しては，効果を示さなかった。