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BACTERIAL AND PHYTOPLASMA DISEASES

Development of a selective medium and antisera for *Pseudomonas syringae* pv. *syringae* from seeds of barley and wheat

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Abstract

Bacterial black node, a disease of barley and wheat, is caused by *Pseudomonas syringae* pv. *syringae* (synonym pv. *japonica*, hereafter *Psj*). Here a selective medium, serine-potassium tellurite-based Psj-selective agar (SPTPsjA) was developed to isolate and detect *Psj*. After 5–7 days on SPTPsjA at 25 °C, characteristic black *Psj* colonies formed with an efficiency equal to that on potato–peptone–glucose agar. Except for *P. viridiflava, P. cichorii*, and some strains of *P. syringae* group bacteria, SPTPsjA either inhibited growth of other phytopathogenic bacteria or enabled their discrimination. To facilitate diagnosis of *Psj*, we prepared three antisera against three strains; in a slide agglutination test, the antisera reacted to all tested strains of *Psj* and failed to react to *P. viridiflava* and *P. cichorii* although some strains of other *P. syringae* group bacteria were indistinguishable from *Psj* with SPTPsjA and antisera. When barley and wheat seeds from fields in which bacterial black node had occurred were placed on SPTPsjA in the dark at 25 °C for 7 days, black colonies grew on the medium around the seeds. Among these strains, only those responsive to the above antisera were pathogenic on barley. These results show that the combination of SPTPsjA and antisera can isolate *Psj* from seeds of barley and wheat.

Keywords Bacterial black node · *Pseudomonas syringae* pv. *syringae* (synonym pv. *japonica*) · Selective medium · Antiserum · Slide agglutination reaction

Introduction

Bacterial black node is a disease of barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) caused by *Pseudomonas syringae* pv. *syringae* (synonym pv. *japonica*, hereafter, *Psj*). Young (1992) reported that *Psj* and *P. syringae* pv. *syringae* were indistinguishable in their biochemical, nutritional, and pathogenic reactions and therefore designated *Psj* as a junior synonym of *P. syringae* pv. *syringae*. This disease emerged as a new bacterial disease after outbreaks occurred around 1945 in Japan over a wide area that included the Seto Inland Sea coastal zone and the Tokai region (Ikata and Hori 1950; Mukoo and Tsuchiya 1950). The ear burn of barley reported by Goto and Nakanishi (1951) is also now regarded as a symptom of bacterial black node. The disease inflicted major damage on barley in particular. The incidence of bacterial black node subsequently declined, only to recur in Japan in 1976 and later in the prefectures of Fukuoka, Niigata, Nagano, and Fukui (Aoyagi et al. 1980; Shimizu et al. 1981; Takamatsu 1983; Yokoyama 1976), and emerged thereafter in the Kyushu, Kinki, and Hokuriku regions (Matsuzawa 1987; Oba et al. 1990; Senba et al. 1994). In Kagawa Prefecture in Japan, bacterial black node was also found in 1996 in hull-less barley (*H. vulgare* var. *nudum* Hook. f., cv. Ichibanboshi), causing ear burn and extensive damage.

Psj is transmitted via seeds (Fukuda et al. 1990; Goto and Nakanishi 1951) and infested straw (Kan 1978), but infected seeds are the most important source of inoculum (Kawaguchi et al. 2017, 2018). Therefore, a method to detect seeds infected with pathogenic bacteria is needed for controlling bacterial black node. One such method is use of a selective

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medium to isolate the living pathogen. Several such media suitable for selectively detecting *Pseudomonas* species (including species under former classifications) (Fieldhouse and Sasser 1982; Hasebe et al. 1998; Miyoshi and Tachibana 1994; Sato et al. 1981; Tsushima et al. 1986; Uematsu et al. 1982). In the present study, we devised a method for selective isolation and detection of *Psj* by combining a selective medium, based on already reported selective media, with the slide agglutination method, which enables simple and speedy diagnosis with antiserum prepared from immunized rabbits. Using this detection method, we investigated the percentage of seeds infected with *Psj* in barley and wheat. Part of the results of this work was reported by Mori et al. (1999).

Materials and methods

Test strains

Among 68 strains of plant pathogenic bacteria evaluated during selective medium tests were 13 strains of *Psj* and 26 other *P. syringae* pathovars (Table 1). Although some of the *P. syringae* group bacteria have been identified as different species in recent years, in this study all are described as *P. syringae*. The DNA group of each *P. syringae* strain used in this study was assessed according to the report of Inoue and Takikawa (2006). The pathogenicity of these isolates was confirmed using barley grown in a greenhouse at 25 °C until the internode elongated. A bacterial suspension of about 10^8 colony-forming units (cfu)/ml was injected in the vicinity of the node with a 1-ml syringe. Plants were then incubated again in a greenhouse. Five days after inoculation, isolates in which the nodes were blackened were regarded as pathogenic. Test strains that had been cultured on PPGA (Nishiyama 1978) at 25 °C for 24 or 48 h were suspended in sterile water at an optical density of 0.3 at 600 nm or at a transmittance of 55–75% at 610 nm (~ 10^8 cfu/ml in each case) and diluted appropriately.

Composition of selective medium

Based on the report of Sato et al. (1981) and Tsushima et al. (1986), we chose the following composition for the basal medium: 1.3 g KH_2PO_4, 1.2 g $Na_2HPO_4 \cdot 12H_2O$, 5.0 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, 24 mg $Na_2MoO_4 \cdot 2H_2O$, 15 g agar, 1 l distilled water.

For testing utilization of carbon sources (Table 2) by the isolates, 10 g of each test substance was added to a liter of the basal medium. This test solution was then autoclaved at 115 °C for 15 min. After that about 10 ml of the solution was dispensed into 90-mm Petri dishes to prepare the agar plates. To test the selectivity of each medium, 100 µl of approximately 10^3 cfu/ml of the bacterial suspension was spread on the plates, using two plates per strain. After incubating at 26 °C in the dark for 4 days, the number of colonies formed on the medium was counted, and colony diameters were measured. The bacterial growth on each medium was compared with that on King's B medium (Eiken Chemical, Tokyo, Japan) because the composition is simple, and the nutrients are commonly used (peptone and glycerin).

Growth-inhibiting substances such as antibiotics were added to PPGA and their effectiveness evaluated (Table 2). Test substances were selected based on the report of Wakimoto and Uematsu (1993). The amount of each substance used per 1 l is shown in Table 2. Boric acid, sodium taurocholate, sodium dodecyl sulfate, and lithium chloride were added to PPGA before sterilization of the medium at 115 °C for 15 min. Kabicidin, an amount equal to one bottle (100 mg titer) was ground into a fine powder in a mortar, triturated with 10 ml of ethyl alcohol, and added to PPGA before sterilization. The other growth-inhibiting test substances were sterilized by filtration through a filter (pore size: 0.45 µm) and were added to PPGA after it had been autoclaved and cooled in a water bath to 55 °C. Methyl violet was dissolved (10 mg in 2 ml of ethanol), and then 8 ml of sterilized distilled water was added; 1 ml of this solution was added to the medium to achieve a concentration of 1 mg/l. Phenol red was dissolved (0.2 g in 10 ml of 1/20 N NaOH), and 1 ml of the resulting solution was added to the medium to achieve a concentration of 20 mg/l. Cycloheximide and rifampicin were dissolved in ethanol, and the others were dissolved in distilled water and then filtered. About 10 ml of the medium dispensed into 90-mm Petri dishes to prepare the agar plates. To test the selectivity of each medium, 100 µl of approximately 10^3 cfu/ml of the bacterial suspension were spread on the plates, which were incubated at 26 °C in the dark for 3 and 7 days. Colonies were then counted and compared with counts on PPGA medium.

Selectivity of the developed medium

To investigate the growth of a given bacterium on the selective medium we developed, 10 µl of the bacterial suspension (approximately 10^8 cfu/ml) was spotted onto three places on the medium. After 2 weeks, bacterial growth and colony color were evaluated. We made dilution plates of *Psj* and of strains that formed colonies similar to *Psj* in the above test. Test bacteria were suspended in sterilized water and then diluted with 1 mM HEPES buffer (pH 7.0) to approximately 10^3 cfu/ml. We spread 100 µl of each bacterial suspension on the plate, using three plates per strain. After incubating at 25 °C in the dark for 7 days, the colonies were counted, and the average of the three plates was calculated and compared with that on King's B and PPGA. This test was repeated twice.

Table 1 Bacterial strains used in this study

Species and pathovar	Strain	Source[a]	Host or origin	Growth on SPTPsjA[b]	Slide agglutination[c]	*P. syringae* group[d]
Pseudomonas syringae						
pv. *syringae* (*japonica*)[e]	Psj-5	KPAES	Barley	+	+	III
	Psj-20	KPAES	Barley	+	+	III
	Psj-22	KPAES	Barley	+	+	III
	Psj-24	KPAES	Barley	+	+	III
	Psj-42	KPAES	Wheat	+	+	III
	Psj-52	KPAES	Barley	+	+	III
	Psj-106	KPAES	Barley	+	+	III
	Kurofushi①-1-1-1	KPAES	Barley	+	+	III
	76A-7	SPARC	Barley	+	+	III
	301072 PT[f]	MAFF	Barley	+	+	III
	301159	MAFF	Wheat	+	+	III
	301163	MAFF	Barley	+	+	III
	301768	MAFF	Barley	+	+	III
syringae	301430	MAFF	Peach	+	+	III
	301861	MAFF	Lilac	+	+	III
	302086	MAFF	Lilac	+	+	III
actinidiae	302091	MAFF	Kiwi	−	−	II
alisalensis[g]	106156	MAFF	Chinese cabbage	+	+	IB
aptata	301012	MAFF	Sugar beet	+	+	III
	302830	MAFF	Rice	+	+	III
atropurpurea	301017 PT	MAFF	Itarian ryegrass	+	−	IV
castaneae	302088 PT	MAFF	Chestnut	±	−	IA
coronafaciens	301314	MAFF	Oat	+	−	IV
eriobotryae	301062	MAFF	Loguat	+	+	IA
glycinea[h]	301683	MAFF	Soybeen	−	−	IA
lachrymans	301315	MAFF	Cucumber	±	+	IB
maculicola	301174	MAFF	Chinese cabbage	+	−	II
mellea	302304 PT	MAFF	Tobacco	+	−	IB
mori	810001	MAFF	Mulberry	±	−	IB
morsprunorum	301436	MAFF	Peach	−	+	IA
myricae	301464 PT	MAFF	Chinese bayberry	+	−	IA
oryzae	301529 PT	MAFF	Rice	+	+	IV
phaseolicola[h]	301616	MAFF	Common bean	±	−	IA
pisi	301208	MAFF	Pea	+	+	III
sesami	311181	MAFF	Sesame	±	−	IB
solidagae	810053 PT	MAFF	*Solidago altissima*	+	+	III
spinaciae	211666 PT	MAFF	Spinach	±	−	II
striafaciens	301032	MAFF	Oat	+	−	IV
theae	302851	MAFF	Tea	+	−	II
Pseudomonas cichorii						
	301367	MAFF	Lettuce	+	−	
Pseudomonas viridiflava						
	301326	MAFF	Cucumber	+	−	
	301327	MAFF	Cucumber	+	−	
	301343	MAFF	Lettuce	+	−	
Pseudomonas marginaris						
pv. *marginaris*	301330	MAFF	Chinese cabbage	−	−	

Table 1 (continued)

Species and pathovar	Strain	Source[a]	Host or origin	Growth on SPTPsjA[b]	Slide agglutination[c]	*P. syringae* group[d]
	311035	MAFF	Broccoli	–	–	
Acidovorax avenae						
	KA_Aci.ave ①	KPAES	Rice	–	–	
Burkholderia andropogonis						
	302546	MAFF	*Gypsophila paniculata*	–	–	
Burkholderia caryophylli						
	301406	MAFF	Carnation	–	–	
Burkholderia gladioli						
pv. *gladioli*	KA_Bur.gla Onci①	KPAES	*Oncidium*	Br	–	
Burkholderia glumae						
	KA_Bur.glu①	KPAES	Rice	–	–	
Burkholderia plantarii						
	302381	MAFF	Rice	–	–	
	302469	MAFF	Rice	n.t	–	
Clavibacter michiganensis						
subsp. *michiganensis*	301245	MAFF	Tomato	–	–	
	301246	MAFF	Tomato	–	–	
	301247	MAFF	Tomato	–	–	
Pantoea ananatis						
	301714	MAFF	Rice	–	–	
Pectobacterium atrosepticum						
	301629	MAFF	Potato	–	–	
Pectobacterium carotovorum						
subsp. *carotovorum*	301297	MAFF	Eggplant	–	–	
	301298	MAFF	Eggplant	–	–	
Pectobacterium rhapontici						
	301332	MAFF	Onion	–	–	
	301333	MAFF	Onion	–	–	
	301341	MAFF	Onion	–	–	
Ralstonia solanacearum						
	KA_Ral.sol Kal①	KPAES	Kalanchoe	–	–	
Rhizobium radiobacter						
	301278	MAFF	Chrysanthemum	Br	–	
Rhizobium rhizogenes						
	301279	MAFF	*Rosa*	–	–	
	302306	MAFF	*Rosa*	–	–	
Xanthomonas campestris						
pv. *vitians*	KA_Xcv 1-①	KPAES	Lettuce	–	–	
pv. *campestris*	KA_Xcc 1-①	KPAES	Cabbage	–	–	

[a]*KPAES* Kagawa Prefectural Agricultural Experiment Station, *MAFF* Ministry of Agriculture, Forestry and Fisheries, *SPARC* Saga Prefectural Agricultural Reseach Center

[b]Result of bacterial suspension spotted on medium: +, black colonies; ±, gray colonies growing slightly; Br, brown colonies; −; no growth; n.t., not tested

[c]Result of agglutination reaction with Psj-5 antiserum. +, positive reaction, −, negative reaction

[d]Grouping of *Pseudomonas syringae* strain according to the method of Inoue and Takikawa (2006)

[e]Pathogen causing bacterial black node

[g]Recently transferred to *Pseudomonas cannabina*

[h]Recently transferred to *Pseudomonas savastanoi*

[f]*PT* pathotype strain, *T* type strain

Table 2 Testing substances for development of selective medium

Type of substance	Test substances
For carbon source (10 g)[a]	
Monosaccharide	D-glucose, D-xylose, galactose, L-arabinose
Disaccharide	Sucrose, maltose
Trisaccharide	Raffinose
Polysaccharide	L-rhamnose
Glycoside	D-salicin
Alcohols	Glycerin, mannitol, sorbitol, ribitol, adonitol
Amino acids	L-serine, L-proline, L-alanine, L-threonine, L-valine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-isoleucine, glycine, L-methionine, L-arginine, L-tryptophan, L-lysine
Hydroxy acid	Quinic acid
For suppression of growth of other bacteria	
Antibiotics	Bacitracin (10 mg), novobiocin (50 mg), penicillin G (60 mg), ampicillin sodium (10 mg), phenethicillin potassium (50 mg), vancomycin hydrochloride (10 mg), polymyxin B (100 mg), rifampicin (0.5 mg), cycloheximide (50 mg), kabicidin (100 mg)
Proliferation inhibitor	Potassium tellurite (25 mg), 2,3,5-triphenyl tetrazolium chloride (50 mg), boric acid (1.5 g), sodium taurocholate (3.0 g), sodium dodecyl sulfate (0.6 g)
Coloring compound	Methyl violet (1 mg), phenol red (20 mg), crystal violet (50 mg)
Metals	Cadmium chloride (5 mg), lithium chloride (7.0 g)
Fungicide	Cetyltrimethylammonium bromide (10 mg)

[a]Amount added per liter

Antiserum preparation and applicability

Antisera were prepared using the method of Maeda and Tsuchiya (1993). *Psj* strains Psj-5, 76A-7, and Kurofushi ①-1-1-1 were used as immunization sources. After culturing on a King's B plate at 25 °C for 2 days, each strain was suspended in 0.85% (w/v) physiological saline to create solutions of about 10^8 cfu/ml, which were washed twice with saline, centrifuged for 10 min at 5000 rpm, and then frozen and stored at − 20 °C. We immunized rabbits sequentially at 7-day intervals with thawed bacterial suspension at doses of 0.5, 1, 2, 3, 4, and 5 ml, and then collected blood 7 days after the last immunization into a 50 ml round bottom centrifuge tube. The centrifuge tube was capped and kept at 37 °C for 2 h while removing the clot from the wall with a glass rod. After overnight incubation at 4 °C, the supernatant was recovered as antiserum. The prepared antisera were used for the slide agglutination reaction. To carry out this reaction, 30 µl of antiserum diluted 1:30 with 0.85% (w/v) physiological saline were placed onto hemagglutination plates. Bacteria grown on the nutrient medium to be tested were then scraped off with a platinum loop and mixed well into the serum in sufficient quantity to make the serum solution slightly cloudy. After mixing, the presence or absence of agglutination was determined by observing the plates by eye and with a stereomicroscope while tilting the plates back and forth for about 1 min. This test was repeated three times.

Isolation and identification of *Psj* from seeds of barley and wheat

Barley seeds (cv. Ichibanboshi) were collected in 1996, 1997, and 2014 from fields in which bacterial black node had occurred. Wheat seeds (cv. Sanukinoyume) were also collected in 2014. The seeds were placed on plates containing the selective medium and the plates kept in the dark at 25 °C for 7 days. Any bacteria that grew on the surface of the medium around the seeds were then subjected to the slide agglutination reaction with 1:30-diluted Psj-5 antiserum solution. Isolated bacteria were tested for pathogenicity to barley as described above.

Results

Selective medium composition

In tests of *Psj* growth on media with one of 30 types of sugars, glycosides, alcohols, hydroxy acids, or amino acids (Table 2), media containing glucose, galactose, or D-xylose as a monosaccharide; sucrose or maltose as a disaccharide; glycerin, mannitol, or sorbitol as an alcohol; quinic acid as a hydroxy acid; or L-serine, L-proline, or L-alanine as an amino acid yielded colony numbers much the same as those obtained on King's B. However, colony diameters were smaller on media containing D-xylose, maltose, glycerin, or

L-alanine. Among the tested substances, L-serine, L-proline, quinic acid, and sorbitol supported little growth of phytopathogenic bacteria species of the *Pectobacterium, Clavibacter, Xanthomonas*, and *Acidovorax*. On media containing L-serine, *Rhizobium* and *Ralstonia* species either did not grow or formed notably smaller colonies (supplementary Table S1).

As for antibiotics and other additives, the growth of *Psj* was clearly suppressed on media containing novobiocin, penicillin G, polymyxin B, rifampicin, boric acid, sodium dodecyl sulfate, crystal violet, or lithium chloride. Bacitracin, 2,3,5-triphenyl tetrazolium chloride, phenol red, and cetyltrimethylammonium bromide suppressed the growth of *Psj* and all other tested phytopathogenic bacteria. Cycloheximide and kabicidin are antifungal agents and do not affect the growth of *Psj*. Ampicillin sodium, phenethicillin potassium, vancomycin, potassium tellurite, sodium taurocholate, cadmium chloride, and methyl violet allowed *Psj* growth equivalent to that of the control medium while suppressing the growth of several other phytopathogenic bacteria (supplementary Table S2).

On the basis of the above results, we created a selective medium for isolating *Psj* and named it serine-potassium tellurite-based *Psj*-selective agar (SPTPsjA; Table 3).

Table 3 Composition of selective medium to isolate bacterial black rot pathogen

KH_2PO_4	1.3 g	Added after autoclaving (115 °C, 15 min), kept at 55 °C	
$Na_2HPO_4{\cdot}12H_2O$	1.2 g		
$(NH4)_2SO_4$	5 g	Ampicillin sodium	10 mg
$MgSO_4{\cdot}7H_2O$	250 mg	Methyl violet[a]	1 mg
$Na_2MoO_4{\cdot}s2H_2O$	24 mg	Potassium tellurite	25 mg
L-serine	5 g	Cycloheximide	25 mg
Kabicidin[b]	100 mg (titer)		
Agar	15 g		
Distilled water	1 l		

[a]10 mg in 2 ml ethanol is added to 8 ml water; 1 ml of the 10 ml solution is added to medium

[b]Amount equal to one bottle (100 mg titer) is ground to a fine powder in a mortar, triturated with 10 ml of ethanol, then added to medium without filtering

Selectivity on SPTPsjA

All of the *Psj* strains tested grew on SPTPsjA, with characteristic black colonies on the after 5–7 days (Fig. 1a, b). In addition to *Psj*, the phytopathogenic bacteria *P. viridiflava, P. cichorii, Rhizobium radiobacter*, and *Burkholderia gladioli* pv. *gladioli* also grew. The colonies formed by *R. radiobacter* and *B. gladioli* pv. *gladioli* were brown (Fig. 1c) and thus easy to distinguish from the black *Psj* colonies; however, the black colonies of *P. viridiflava* and *P. cichorii* were difficult to differentiate. To investigate the selectivity of the medium, we also tested 147 bacterial isolates (which grew on PPGA medium at 28 °C and were nonpathogenic to barley) from nonsterilized barley seed and four isolates from barley plants. Except for one aberrant strain isolated from a plant (which was considered to *Pseudomonas* sp.), the other strains did not grow on the medium (data not shown). The colony-forming efficiency of *Psj* strains on SPTPsjA varied from 56.2 to 121.7% of that on PPGA medium (Table 4). The colony-forming efficiency of

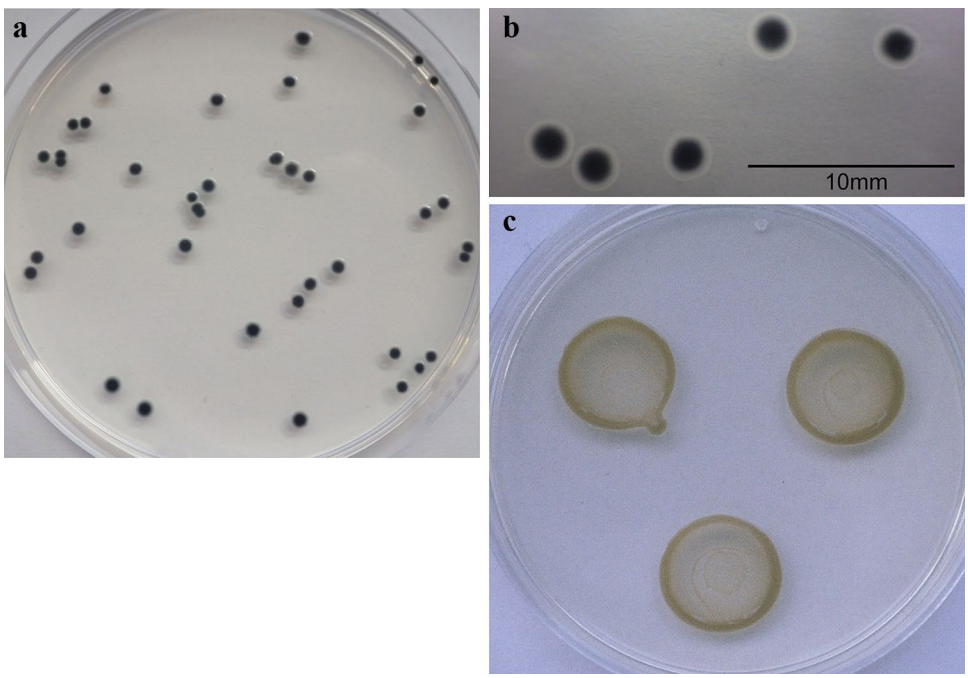

Fig. 1 Colonies of *Pseudomonas syringae* pv. *syringae* (bacterial black node pathogen) (**a, b**) and *Burkholderia gladioli* pv. *gladioli* (**c**) on selective medium SPTPsjA after incubation at 25 °C in the dark for 7 days

strains of *P. syringae* pathovars *syringae, alisalensis, aptata, coronafaciens, lachrymans, mellea, pisi, phaseolicola, sesami, solidagae*, and *theae* was similar to that of the *Psj* strains. The colonies of *P. syringae* pv. *phaseolicola* and *sesami* were very small (< 0.1 mm diameter). Other strains of *P. syringae* either did not grow or only grew slightly on SPTPsjA.

Applicability of antisera

The antiserum for Psj-5, 76A-7, and Kurofushi ①-1-1-1 at 1:30 dilution yielded positive slide agglutination reactions (supplementary Fig. S1) with all tested *Psj* isolates and negative reactions with *P. viridiflava* and *P. cichorii* (Table 4). Of the 26 isolates of *P. syringae* pathovars, 17 isolates had positive reactions, and nearly all reacted with two or all three of the antisera—albeit with slight differences in the strength of the positive reactions. The use of SPTPsjA with the slide agglutination reaction differentiated *Psj* except for a positive result also for *P. syringae* pathovars *syringae, alisalensis, aptata, pisi*, and *solidagae*. We classified all these pathovars except pv. *alisalensis* into group III by genetic grouping (Table 4). In tests of the agglutination reaction of the other strains with the Psj-5 antiserum, the 1:30 dilution did not react with any of the phytopathogens other than *P. syringae* (Table 1) or with the nonpathogenic bacteria isolated from barley seeds (data not shown).

Detection of *Psj* in seeds of barley and wheat

Barley seeds were collected from two fields in 1996; when these seeds were placed on SPTPsjA plates, bacteria grew around 38 of 40 seeds from one field and 54 of 60 seeds from the other. Among the bacteria isolated from the seeds, 19 and 38 isolates from the respective fields reacted to the antiserum of Psj-5. Twenty-five of these isolates were then used in a pathogenicity test; 15 reacted to the antiserum and were pathogenic to barley, and the other 10 did not react with the antiserum and were not pathogenic. From 100 barley seeds collected from each of three fields in 1997, bacteria grew on SPTPsjA from 52, 57, and 57 seeds, respectively, and 12, 16, and 6 of these isolates reacted to the Psj-5 antiserum. In 2014, bacteria grew from 93 to 92 seeds of 100 seeds each from two fields, and 86 and 92 of these isolates reacted to the Psj-5 antiserum. For 100 wheat seeds collected in 2014 from each of four fields, 17, 13, 65, and 58 seeds yielded bacteria on the selective medium, and 15, 9, 54, and 49 of the respective isolates reacted to the Psj-5 antiserum.

Discussion

Tsao (1970) stated that a selective medium can have three effects to select for a target bacterium: selective growth promotion of the target bacterium by an amendment that is utilized specifically by the bacterium, selective growth inhibition of the nontarget bacteria, and selective identification by adding a substance only the target bacterium uses to produce a characteristic indicator pigment. Although Hasebe et al. (1998) reported a selective medium for *Psj*, they suggested that it be refined to improve its specificity. They attributed that medium's lack of specificity to the use of peptones (a mixture of various organic compounds), and the use of glycerin, a substance used by many different bacteria as a carbon source. To overcome these problems, we prepared a completely synthetic medium, and the single carbon source, L-serine, increased selectivity. Also, of various substances we tested for their selective inhibition potential, we chose ampicillin sodium and methyl violet—both have been used in media selective for *Pseudomonas* spp. and *Burkholderia glumae* (Fieldhouse and Sasser 1982; Hasebe et al. 1998; Miyoshi and Tachibana 1994; Sato et al. 1981; Tsushima et al. 1986; Uematsu et al. 1982). These substances did not inhibit *Psj* multiplication but did inhibit the multiplication of other phytopathogenic bacteria and nonpathogenic bacteria isolated from barley. The substance with the greatest selective inhibition of bacteria other than *Psj* was potassium tellurite, used in a selective medium for *P. cichorii* created by Uematsu et al. (1982), but allowed the growth of black *Psj* colonies, thereby aiding selective identification as well as selective inhibition. To complete the medium, we also added cycloheximide and kabicidin, which suppress filamentous fungi; kabicidin, an antibiotic extracted from *Streptomyces gougerotii* culture liquid, only suppresses the growth of true fungi.

Because we were unable to completely suppress growth of *P. viridiflava* and nonpathogenic bacteria, we also prepared antisera by using *Psj* strains as antigens and used these antisera to test isolates that grew on the selective medium by the slide agglutination method. The antiserum reacted to some of the *P. syringae* strains, including all *Psj* strains. By combining the slide agglutination method with the selective medium, we could specifically detect *Psj* in barley and wheat seeds. On the other hand, six strains of *P. syringae* pathovars *syringae, alisalensis, aptata, pisi*, and *solidagae* grew on SPTPsjA and reacted with the antisera in the slide agglutination reaction test just as *Psj* did (Table 4). Except for one strain (pv. *alisalensis*), these strains are in the same genetic group (group III) and thus cannot be identified by molecular techniques such as PCR. It is important to easily isolate living pathogenic

Table 4 Dilution plate efficiency for selective medium and bacterial response to three antisera

		SPTPsjA/PPGA(%)[a]		Slide agglutination[b]			*P. syringae* group[c]
		rep.1	rep.2	Psj-5	76A-7	Kurofushi①-1-1-1	
Pseudomonas syringae pathovars							
syringae (*japonica*)[d]	Psj-5	81.1	76.2	+	+	+	III
	Psj-20	75.0	65.3	+	+	+	III
	Psj-22	62.6	68.5	+	+	+	III
	Psj-24	56.2	81.5	+	+	+	III
	Psj-42	106.7	69.9	+	+	+	III
	Psj-52	75.4	106.5	+	+	+	III
	Psj-106	75.3	102.4	+	+	+	III
	Kurofushi①-1-1-1	78.5	74.2	+	+	+	III
	76A-7	121.7	105.4	+	+	+	III
	301072	83.7	87.4	+	+	+	III
	301159	87.0	78.4	+	+	+	III
	301163	89.8	91.9	+	+	+	III
	301768	n.t	n.t	+	+	+	III
syringae	301430	75.9	99.2	+	D	+	III
	301861	124.0	76.2	+	D	+	III
	302086	91.4	71.8	+	+	+	III
actinidiae	302091	0.0	0.0	−	−	−	II
alisalensis[e]	106156	72.2	43.6	+	+	+	IB
aptata	301012	81.3	79.3	+	+	+	III
aptata	302830	99.4	77.8	+	+	+	III
atropurpurea	301017	2.0	0.3	D	D	+	IV
castaneae	302088	0.0	0.2	d	d	d	IA
coronafaciens	301314	77.3	54.8	−	−	d	IV
eriobotryae	301062	0.0	0.0	d	d	−	IA
glycinea[f]	301683	0.0	0.0	D	D	−	IA
lachrymans	301315	100.8	91.5	d	d	−	IB
maculicola	301174	8.5	28.6	−	−	−	II
mellea	302304	76.4	90.7	−	−	−	IB
mori	810001	0.0	0.0	D	D	−	IB
morsprunorum	301436	0.0	0.0	d	d	d	IA
myricae	301464	7.8	17.2	−	−	−	IA
oryzae	301529	10.6	5.5	+	+	+	IV
phaseolicola[f]	301616	101.8	93.0	−	−	d	IA
pisi	301208	89.5	80.1	+	+	+	III
sesami	311181	78.6	76.9	−	−	−	IB
solidagae	810053	97.5	80.2	+	+	+	III
spinaciae	211666	0.0	0.0	−	−	−	II
striafaciens	301032	30.0	21.9	−	−	d	IV
theae	302851	156.8	142.5	−	−	−	II
Pseudomonas cichorii	301367	22.2	54.0	−	−	−	−
Pseudomonas viridiflava	301326	121.1	120.4	−	−	−	−
	301327	97.5	125.7	−	−	−	
	301343	108.1	97.9	−	−	−	

[a]No. of colonies on SPTPsjA/No. of colonies on PPGA × 100; n.t., not tested

[b]Test was done three times; +, three positive reactions; D, two positive and one negative; d, one positive and two negative; −, three negative reactions

[c]Grouping of *Pseudomonas syringae* strain according to the method of Inoue and Takikawa (2006)

[d]Pathogen causing bacterial black node

[e]Recently transferred to *Pseudomonas cannabina*

[f]Recently transferred to *Pseudomonas savastanoi*

bacteria using selective medium because identification of *Psj* depends on confirming pathogenicity to wheat or barley by inoculation test. A PCR method is effective for rapid and high-sensitivity detection of the pathogen without isolation (Yoshioka et al. 2014). These various methods can be useful depending on the intended purpose. Although a PCR method can be used to identify bacteria grown on SPTPsjA medium, we used the slide agglutination reaction here because it produces results in a few minutes and does not require equipment such as a thermal cycler.

Because seeds infected with *Psj* are the most important source of inoculum for bacterial black node (Kawaguchi et al. 2017), control of the disease depends largely on determining whether seeds are infected. Our study revealed that the proportion of seeds infected with *Psj* varies widely from year to year in barley and, depending on the field, even in the same year in wheat. Unfortunately, all seeds collected from fields were infected with *Psj*. In contrast, barley and wheat seeds from plants cultivated in the greenhouse are rarely infected with *Psj* (data not shown). Fukuda et al. (1990) confirmed that *Psj* cells enter through pores in the epidermis of the lemma and multiply in the intercellular spaces in parenchymal tissue. The weather (rain and wind) during the time when *Psj* can infect seeds (such as at flowering stage) can strongly influence bacterial colonization in seed. In the future, we need to study the interrelationship among weather factors, seed infections, and the occurrence of bacterial black node disease.

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Compliance with ethical standards

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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