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METHODS AND PROTOCOLS

Three semi-selective media for *Pseudomonas syringae* pv. *maculicola* and *P. cannabina* pv. *alisalensis*

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Abstract

Three semi-selective media, DTarTA, SPbc, and SPamt, were developed and tested to isolate *Pseudomonas syringae* pv. *maculicola* (*Psm*) and *P. cannabina* pv. *alisalensis* (*Pca*) from *Raphanus sativus* seeds. DTarTA contained D-tartaric acid as a carbon source and potassium tellurite, ampicillin sodium, and methyl violet as antibiotics. DTarTA suppressed growth in 19 of the 24 pathovars from the *P. syringae* complex, whereas *Psm* and *Pca* grew and formed gray to black colonies. SPamt contained sucrose and peptone as nutrient sources and was supplemented with bromothymol blue and the same antibiotics present in DTarTA and *Psm* and *Pca* formed yellowish to dark brown colonies on the SPamt medium. SPbc contained sucrose and peptone and was supplemented with cephalexin and boric acid as antibiotics and *Psm* and *Pca* formed semi-translucent to white colonies on the SPbc medium. SPamt and SPbc suppressed the growth of several plant-associated bacteria (except the *P. syringae* complex). The growth of saprophytic bacteria in seeds on the different media was compared with that on King's B medium, using five types of commercially available *Raphanus sativus* seeds. The suppression rate of DTarTA was 85–99% and was lower for seeds with more saprophytic bacteria. The suppression rates of SPamt and SPbc were 90–99%. In detection tests using 10,000 seed samples mixed with *Pca* or *Psm*-contaminated seeds, it was possible to selectively isolate *Psm* and *Pca* using SPamt and SPbc, even when the colony numbers of the target bacterium constituted less than 10% of the total colonies.

Key points

- *Bacterial leaf spot and blight pathogens were selectively isolated from seeds.*
- *DTarTA medium distinguishes these pathogens from P. syringae complex pathovars.*
- *SPamp and SPbc media have different selectivity for plant-associated bacteria.*

Keywords Bacterial leaf spot · Bacterial leaf blight · Specific detection · Semi-selective medium · *Pseudomonas syringae* complex · *Raphanus sativus* seed

Introduction

Bacterial leaf spot and leaf blight are global threats to the cultivation of cruciferous vegetables. *Pseudomonas syringae* pv. *maculicola* (*Psm*) causes bacterial leaf spots in plants such as cabbage, cauliflower, broccoli, Chinese cabbage, turnip, radish, and Japanese radish (Peters et al. 2004; Takikawa and Takahashi 2014; Zhao et al. 2000) and was first reported by McCulloch (1911). *Psm* is closely resembles pathovar *tomato* in bacteriological characteristics and genetic similarity (Peters et al. 2004; Wiebe and Campbell 1993). Both pathogens can infect each other's hosts when artificially inoculated (Hendson et al. 1992; Takikawa et al. 1994; Wiebe and Campbell 1993). It was reported that there are at least eight genetic lineages among *Psm* and pathovar tomato isolates and there is a relationship between the genetic lineages and isolation sources (Gironde and Manceau 2012). *Psm* also closely resembles pathovar *spinaciae* (Bazzi et al. 1988; Inoue and Takikawa 2021; Koike et al. 2002; Ozaki et al. 1998). *Psm* are classified into four groups (I–IV) according to their bacteriological characteristics and the host from which they are isolated (Takikawa and Takahashi 2014; Takikawa et al. 1994). Group I consists

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of the majority of worldwide isolates, including pathovar *tomato*. Group II consists of various Japanese radish isolates, which cause small leaf spots and internal root discoloration. Group III mainly consists of *Brassica rapa* isolates from Japan. Group IV consists of various Japanese radish isolates which cause large necrotic lesions (now reclassified as a different pathotype). *Pseudomonas cannabina* pv. *alisalensis* (*Pca*) causes bacterial leaf blight in plants such as arugula, cabbage, Chinese cabbage, radish, and bristle oat (Bull et al. 2004; Ishiyama et al. 2013; Mauzey et al. 2011; Rubio et al. 2012; Sarris et al. 2010; Takahashi et al. 2013; Wechter et al. 2010) and was first reported by Cintas et al. (2002). Some strains that were isolated and identified as *Psm* (group IV) have now been reclassified as *Pca* (Rubio et al. 2012; Takahashi et al. 2013; Takikawa and Takahashi 2014). *Pca* is classified into two types (A and B) based on differences in host virulence and genetic diversity (Sarris et al. 2013; Takikawa and Takahashi 2014). They were divided based on utilization tests of sorbitol, malonate, caprate, and acetate. *Psm* and *Pca* invade the stomata of leaves and plant wounds, creating small chlorotic and necrotic spots with water-soaked regions on the leaves, and cause severe blight symptoms (Peters et al. 2004; Takikawa and Takahashi 2014; Zhao et al. 2000). Infections by *Psm* and *Pca* cause black brown discoloration in the core of the stem root and bacterial spots on the surface of the stem root of Japanese radish, which is a major problem in Japan (Horinouchi et al. 2009; Inoue and Takikawa 2021; Otani 2016; Takeuchi et al. 1989). Similar to other bacteria in the *P. syringae* group, both *Psm* and *Pca* are considered seed-borne (Schofield et al. 2012; Takimoto 1931). Therefore, the distribution of seeds contaminated with these pathogens is thought to play an important role in the occurrence of disease.

In general, tests to infer the pathogen-free status of seeds require the isolation of live pathogenic bacteria. Thus, a selective medium with high selectivity for the target pathogenic bacterium is required. KBC medium (Mohan and Schaad 1987) is widely used for the isolation of bacteria in the *P. syringae* group (Asaad et al. 2017; Bull and Koike 2017; Randhawa et al. 2017; Shepherd and Block 2017). However, KBC has been developed to isolate *P. syringae* pv. *syringae* from beans, and it is difficult to distinguish between the saprophytic bacteria and target bacteria, *Psm* and *Pca* based on their colony morphology in this medium; furthermore, the suppression of saprophytic bacteria by this medium is imperfect. Thus, the development of selective media that suppress the growth of more saprophytic bacteria and allow *Psm* and *Pca* to form colonies with distinctive morphological characteristics would help in the isolation of these pathogens. KBC contains cephalexin and boric acid which act as antibacterial substances in King's B medium (KB; King et al. 1954). By changing the composition of the antibacterial component, it may be possible to prepare a new medium that differs from KBC in terms of its selectivity to saprophytic bacteria. MSP (Mohan and Schaad 1987) and SPTPsjA (Mori et al. 2018) media have also been developed as semi-selective media to isolate bacteria in the *P. syringae* group. MSP uses sucrose as its main carbon source and is suitable for separating the *P. syringae* complex from other *Pseudomonas* spp. SPTPsjA enhances the selection of *P. syringae* pv. *syringae* using L-serine as the sole carbon source and contains multiple antibiotics. By combining the characteristics of these media, it may be possible to develop new selective media. In this study, three semi-selective media for *Psm* and *Pca* were developed based on the aforementioned media.

Materials and methods

Bacterial strains and culture conditions

Sixty-five strains of plant-associated bacteria were used in these experiments (Table 1). The *Pseudomonas* strains, including five strains of *Pca*, eight strains of *Psm*, and 23 other strains belonging to the *P. syringae* group, as well as an *Acidovorax* strain, were cultured on PPGA medium (Nishiyama 1978) at 25 °C. The *Xanthomonas* and *Ralstonia* strains were cultured on potato semisynthetic medium (PSA) (Wakimoto 1960) at 27 °C, and those in the *Erwinia* group and *Burkholderia* strains were cultured on yeast-peptone agar medium (YPA: 5 g yeast extract, 10 g peptone, 15 g agar, and 1000 mL of distilled water; pH 6.8) at 27 °C. These cultures were suspended in sterile distilled water at an optical density of 0.3 at 600 nm (~ 10^8 CFU/mL in each case) and appropriately diluted for subsequent examinations. Rifampicin-resistant strains KMrR-R03 (derived from MAFF (Ministry of Agriculture, Forestry and Fisheries, Japan) 106,179) and NMH-R1 (derived from MAFF 106,156) (Inoue and Takikawa 2021) were used to create pathogen-infected seeds.

D-tartaric acid, tellurite, and ampicillin medium

To the base of the SPTPsjA (KH_2PO_4: 1.3 g; $Na_2HPO_4 . 12H_2O$: 1.2g; $MgSO_4 . 7H_2O$: 0.25 g; $(NH_4)_2SO_4$: 5 g) medium, 5 g of D-tartaric acid was added. This was dissolved in 1 L of distilled water, and the pH was adjusted to 6.8. Fifteen grams of agar was added, and the medium was autoclaved at 115 °C for 15 min and then cooled to approximately 50 °C. To this, 100 µL each of ampicillin sodium (100 mg/mL in sterile water), potassium tellurite (250 mg/mL in sterile water), methyl violet (10 mg/mL in 70% ethanol), and cycloheximide (100 mg/mL in 70% ethanol) was added. The medium was mixed well. Approximately 15 mL

Table 1 Bacterial strains

Species and pathovar			Strain name	Souce	Host plant	Growth		
						D-TarTA	SPamt	SPbc
Pseudomonas								
	cannabina	*alisalensis*	106156[a]	MAFF	Chinese cabbage	+[b]	+	+
			212064	MAFF	bristle oat	+	+	+
			212402	MAFF	*Brassica rapa* (Komatsuna)	+	+	+
			301419	MAFF	Japanese radish	+	+	+
			730087	MAFF	Japanese radish	-	+	+
	coronafaciens	*atropurpurea*	301017	MAFF	Itarian ryegrass	w	+	+
		coronafaciens	301314	MAFF	oat	w	+	+
		oryzae	301529	MAFF	rice	w	+	+
		striafaciens	301032	MAFF	oat	-	+	+
	savastanoi	*glycinea*	301683	MAFF	soybeen	-	+	+
		phaseolicola	301616	MAFF	bean	-	+	-
	syringae	*actinidiae*	302091	MAFF	kiwi	w	w	-
		aptata	301012	MAFF	sugar beet	-	+	+
		castaneae	302088	MAFF	*Castanea crenata*	-	+	+
		eriobotryae	301062	MAFF	loguat	-	w	-
		lachrymans	301315	MAFF	cucumber	w	+	+
		maculicola	106179	MAFF	Japanese radish	+	+	+
			301174	MAFF	Chinese cabbage	+	+	+
			301175	MAFF	Chinese cabbage	+	+	+
			302539	MAFF	Chinese cabbage	+	+	+
			302723	MAFF	Japanese radish	+	+	+
			302783	MAFF	turnip	+	+	+
			730088	MAFF	Japanese radish	+	+	+
			1331[c]	SUPP	cauliflower	+[d]	+[d]	+[d]
		mellea	302304	MAFF	tobacco	+	+	+
		mori	810001	MAFF	mulberry	-	w	+
		morsprunorum	301436	MAFF	Japanese apricot	-	w	+
		myricae	301464	MAFF	Japanese bay berry	-	+	-
		pisi	301208	MAFF	pea	-	+	+
		sesami	311181	MAFF	sesame	-	+	+
		solidagae	810053	MAFF	*Solidago canadensis*	w	+	-
		spinaciae	211666	MAFF	spinach	+	+	+
		syringae	301861	MAFF	lilac	-	+	+
			301430	MAFF	peach	-	+	+
		theae	302851	MAFF	tea	-	+	+
		tomato	302665	MAFF	tomato	+	+	+
	cichorii		301367	MAFF	lettuce	+	+	-
	fuscovaginae		106631	MAFF	rice	w	+	+
	viridiflava		301327	MAFF	cucumber	+	+	+
			301343	MAFF	lettuce	+	+	+
	(*fluorescens*)[e]		2779[f]	JCM		+	w	-
			20030	JCM		-	-	-
			20064	JCM		w	-	-
			3081[g]	NBRC		w	-	-
			12180	NBRC		w	-	-
			12568	NBRC		-	-	-
			13922	NBRC		+	-	w

Table 1 (continued)

Species and pathovar			Strain name	Souce	Host plant	Growth		
						D-TarTA	SPamt	SPbc
Pseudomonas								
			14808	NBRC		+	-	-
			101042	NBRC		+	-	+
			101043	NBRC		+	-	+
Acidovorax								
	avenae subsp. *avenae*		106618	MAFF	rice	+	-	-
Burkholderia								
	andropogonis		302667	MAFF	corn	-	-	w
	gladioli	*gladioli*	106671	MAFF	rice	+	-	w
	glumae		106747	MAFF	rice	w	-	w
	plantarii		106721	MAFF	rice	w	-	w
Dickeya								
	dadantii		106634	MAFF	sweet potato	-	-	-
	zeae		212010	MAFF	Japanese ginger	-	-	-
Pantoea								
	ananatis		301722	MAFF	rice	-	-	-
Pectobacterium								
	carotovorum subsp. *carotovorum*		301941	MAFF	ginger	-	-	-
Ralstonia								
	pseudosolanacearum		107632	MAFF	tomato	-	-	-
Xanthomonas								
	axonopodis	*vitians*	301289	MAFF	lettuce	-	-	w
	campestris	*campestris*	301176	MAFF	cabbage	-	-	-
		raphani	106181	MAFF	Japanese radish	-	-	-
	oryzae	*oryzae*	311018	MAFF	rice	-	-	-
	vesicatoria		301256	MAFF	tomato	-	-	-

[a] Collection number in the Ministry of Agriculture, Forestry and Fisheries (MAFF) culture collection

[b] +, Positive; -, negative; w, weak growth

[c] Collection number in the Shizuoka University Plant Pathology (SUPP) culture collection

[d] Result of the dilution plating method

[e] Strain isolated as *P. fluorescens*

[f] Collection number in the Microbe Division of Riken Bioresource Research Center (JCM) culture collection

[g] Collection number in the Biological Resource Center of the National Institute of Technology and Evaluation (NBRC) culture collection

of the medium was added to a 9-cm sterile Petri dish to prepare a plate medium.

Sucrose-peptone medium with ampicillin, methyl violet, and potassium tellurite

The determined composition (KH_2PO_4: 1 g; $MgSO_4 \cdot 7H_2O$: 0.2 g; Bacto-peptone: 5 g; sucrose: 20 g) was dissolved in 1 L of distilled water; 1 mL of bromothymol blue (BTB, dissolved in ethanol at 50 mg/mL) was added; and the pH was corrected to 6.2, 6.4, 6.6, 6.8, and 7.0. Fifteen grams of agar was added to this, and the mixture was autoclaved at 121 °C for 20 min. The autoclaved medium was cooled to approximately 50 °C. To this, 100 µL each of ampicillin sodium (100 mg/mL in sterile water), potassium tellurite (250 mg/mL in sterile water), and methyl violet (10 mg/mL in 70% ethanol), as well as 3.5 mL of nystatin (10 mg/mL in 70% ethanol), were added. The medium was mixed well, and approximately 15 mL of it was added to a 9-cm sterile Petri dish to prepare a plate medium.

Sucrose-peptone medium with boric acid and cephalexin

The basic composition of the medium (KH_2PO_4: 1 g; $MgSO_4 \cdot 7H_2O$: 0.2 g; Bacto-peptone: 5 g; sucrose: 20 g) was the same as that used for the *s*ucrose-*p*eptone medium with

*a*mpicillin, *m*ethyl violet and potassium *t*ellurite (SPamt). The components were dissolved in 900 mL of distilled water. The pH was adjusted to 7.0 because the addition of boric acid after sterilization lowered the pH. Fifteen grams of agar was added, and the medium was autoclaved at 121 °C for 20 min and then cooled to approximately 50 °C. To this, 100 mL of boric acid (1.5 g/mL in sterile water), 8 mL of cephalexin (10 mg/mL in sterile water), and 3.5 mL of nystatin (10 mg/mL in 70% ethanol) were added. The medium was mixed well, and approximately 15 mL of it was added to a 9 cm sterile petri dish to prepare a plate medium.

Growth test of plant-associated bacteria on DTarTA, SPamt, and SPbc

Suspensions of all the bacterial strains shown in Table 1, except the *Psm* strain SUPP (Shizuoka University Plant Pathology) 1331, were prepared. A suspension of eight bacterial strains was added to each well in the horizontal rows starting in the left top corner of a 96-well plate. The second, fourth, and sixth rows were not used. A suspension of the bacterial strains was added to each of the eight wells in the third, fifth, and seventh row. A total of 32 strains were used per plate. The volume of each bacterial suspension was 150 µL. The pins of a copy plate (TK-CP96-1/2; Tokken, Chiba, Japan) were inserted into the wells to attach the bacterial suspensions, and the copy plate was then placed on the medium. The copy plate was removed after 10 s, and the surface of the medium was allowed to dry (with the cover open) for 5 min on a clean bench. The medium was then incubated at 25 °C. Bacterial growth was evaluated after 3, 5, and 7 days for *D-tar*taric acid, *t*ellurite, and *a*mpicillin (DTarTA) and after 3 and 5 days for SPamt and *s*ucrose-*p*eptone medium with *b*oric acid and *c*ephalexin (SPbc). The tests were performed twice.

To compare the plating efficiencies of diluted media, six strains of *Psm* and four strains of *Pca* were used. The bacterial suspensions were diluted with 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.0) to approximately 10^3 CFU/mL. Using three plates per strain, 75 µL of each diluted bacterial suspension was spread on the plate. After incubating at 25 °C in the dark for 3 and 5 days, the number of colonies formed on the medium was counted and the average for the three plates was calculated in terms of strain, dilution, and incubation period. The bacterial growth on these media was compared to those on KB and KBC. This test was repeated three times, and differences in the number of colonies between media for each strain were evaluated using Tukey's HSD test.

Evaluation of the media for the inhibition of saprophytic bacteria associated with *Raphanus sativus* seeds

For this experiment, commercially available *Raphanus sativus* seeds that had not been seed-treated were used. The five seed types were categorized as A (Japanese radish of unknown variety, provided by the Center for Seeds and Seedlings (NARO)), B (Hataro, Takii Seeds, Kyoto, Japan), C (Cyoukouaonaga, Takii Seeds), D (Sakuranbo, Sakata Seeds, Kanagawa, Japan), and E (Yukikomachi, Sakata Seeds). Bacteria were extracted from the seeds using the same method as that used for black rot (Export and International Affairs Bureau 2016; International Seed Testing Association 2017). The weight of 100 seeds was measured 10 times, the average weight was calculated, and 100 times the average weight of the 100 seeds was recorded as the weight of 10,000 seeds. Seed sets were placed in 500 mL or 1000 mL conical flasks. When the weight of 10,000 seeds was 100 g or less, a 2.5 × volume of wash buffer (0.85% NaCl, 0.02% Tween20) of the seed weight was added. When the weight was 100 g or more, a 2.25 × volume of buffer of the seed weight was added. The sample was shaken at 120 rpm for 2.5 h, and then 10 mL of the supernatant was collected. The seed extract was diluted using tenfold dilution steps, and 100 µL of the diluted solution

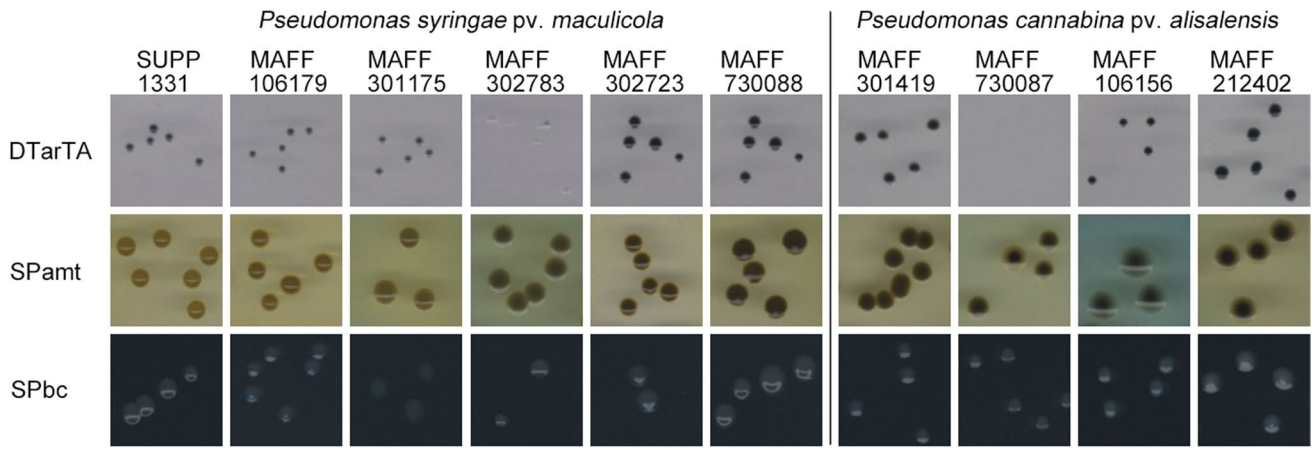

Fig. 1 Colonies of *Pseudomonas syringae* pv. *maculicola* and *P. cannabina* pv. *alisalensis* on DTarTA, SPamt, and SPbc media after 5, 3, and 3 days, respectively. Each image shows a 1 × 1 cm^2 area

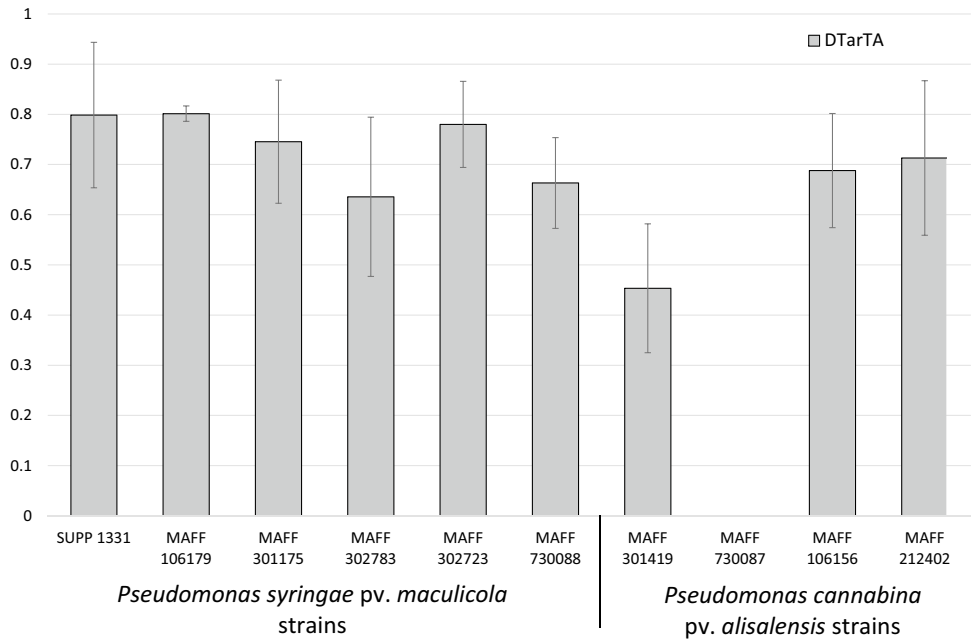

Fig. 2 Ratio of the number of colonies of *Pseudomonas syringae* pv. *maculicola* and *P. cannabina* pv. *Alisalensis* grown on DTarTA medium to that on KB medium. The vertical bars indicate the standard error for three independent experiments

from each step was spread on two plates each of DTarTA, SPamt, SPbc, KBC, and KB media. The number of colonies formed in the medium was counted seven days after inoculation. The suppression rate was determined from the ratio of the number of colonies in the experimental medium to the number of colonies in the KB medium (Mohan and Schaad 1987).

Recovery of *Psm* and *Pca* from seed samples containing inoculated seeds

The infected seeds were inoculated with KMrR-R03 and NMH-R1 (Inoue and Takikawa 2021). The seeds were infected with approximately 0–500 CFUs of the inoculated strain per seed. Seed sets were mixed with either 50 *Pca*-inoculated seeds or 50 *Psm*-inoculated seeds to a total of 10,000 seeds per set, and seed extracts were prepared from these seed sets. The seed extract was diluted in 1 mM HEPES buffer (pH 7.0). Finally, 100 µL each of the undiluted extract, a tenfold diluted solution, and a 100-fold diluted solution were applied to two plates of each medium. To measure the density of target bacteria in the liquid, 100 µL of the undiluted extract was applied to two plates of YPA medium containing 20 mg/L of rifampicin and 35 mg/L of nystatin, and the number of colonies was counted after 3 days. As a control (to observe the morphological characteristics of colonies for colony identification), 100 µL of a suspension of KMrR-R03 and NMH-R1 (diluted to approximately 10^3 CFU/mL) was also added to the medium. After 5–7 days for DTarTA and 3–5 days for SPamt, SPbc, and KBC, colonies were transferred from the medium surface with a sterile toothpick to YPA containing 20 mg/L rifampicin. At that time, the colonies judged to be similar to those of the reference strain based on morphological characteristics like size, color, surface appearance, edge,

Table 2 Reduction in the population of saprophytic bacteria isolated from different sets of *Raphanus sativus* seeds on the semi-selective agar medium

	CFU of saprophytic bacteria /mL of extract								
	3 days after plating			5 days after plating			7 days after plating		
Seed set	KB	KBC	DTarTA	KB	KBC	DTarTA	KB	KBC	DTarTA
A	2.00×10^5	990 (99.5)	2.05×10^4 (89.7)	2.00×10^5	8.35×10^4 (58.1)	2.32×10^4 (88.4)	2.00×10^5	8.45×10^4 (57.6)	2.44×10^4 (87.7)
B	1.36×10^4	1.12×10^3 (91.8)	2.02×10^3 (85.2)	1.44×10^4	1.16×10^3 (91.9)	2.04×10^3 (85.8)	1.46×10^4	1.20×10^3 (91.8)	2.06×10^3 (85.8)
C	4.10×10^4	20 (99.9)	0 (100)	4.15×10^4	35 (99.9)	0 (100)	4.15×10^4	140 (99.7)	0 (100)
D	1.26×10^4	150 (98.8)	180 (98.6)	1.59×10^4	280 (98.2)	195 (98.8)	1.65×10^4	345 (97.9)	200 (98.8)
E	2.1×10^5	1.63×10^4 (92.1)	2.82×10^4 (86.4)	2.09×10^5	2.19×10^4 (89.5)	2.95×10^4 (85.9)	2.1×10^5	2.41×10^4 (88.5)	2.99×10^4 (85.7)

and elevation were noted. The colonies that grew 2–3 days after subculturing were considered to be the target strain, and the ratio of target strain colonies to all colonies was calculated. The proportion of target bacteria that could or could not be predicted as target bacteria at the time of colony transfer was also calculated. Using some of the isolated colonies, a specific PCR test was performed on *Psm* and *Pca* (Inoue and Takikawa 2021) to confirm that the bacteria were indeed *Psm* or *Pca*.

Results

Growth of Psm and Pca on DTarTA

The growth of plant-associated bacteria on DTarTA was evaluated. In the *P. syringae* complex, *Psm*, *Pca*, and *P. syringae* pvs. *mellea*, *spinaciae*, and *tomato* grew on DTarTA (Table 1). *Psm*

Table 3 Isolation of *Pseudomonas cannabina* pv. *alisalensis* and *P. syringae* pv. *maculicola* from 5 different sets of *Raphanus sativus* seeds using DTarTA

Target bacterium + A2:F32	Set of seed	Total number of bacterial colonies	Number of target bacterial colonies	Identified as a target bacterium	Misidentified as a target bacterium
Pseudomonas cannnabina pv. *alisalensis*	A	112	1 (0.89)	0 (0.00)	32
		93	1 (1.08)	0 (0.00)	7
		43	2 (4.65)	0 (0.00)	3
	B	65	20 (30.8)	19 (95.0)	0
		54	15 (27.8)	14 (93.3)	1
		30	15 (50.0)	15 (100)	0
	C	10	10 (100)	10 (100)	0
		11	11 (100)	11 (100)	0
		4	4 (100)	4 (100)	0
	D	45	4 (8.89)	4 (100)	32
		45	1 (2.22)	1 (100)	21
		21	3 (14.3)	2 (66.7)	13
	E	91	2 (2.20)	0 (0.00)	0
		86	1 (1.16)	0 (0.00)	0
		80	2 (2.50)	0 (0.00)	0
Pseudomonas syringae pv. *maculicola*	A	140	0 (0.00)	0 (0.00)	4
		118	1 (0.85)	0 (0.00)	8
		70	2 (2.86)	0 (0.00)	3
	B	43	18 (41.9)	18 (100)	0
		46	20 (43.5)	20 (100)	0
		18	9 (50.0)	9 (100)	0
	C	5	5 (100)	5 (100)	0
		4	4 (100)	4 (100)	0
		3	3 (100)	3 (100)	0
	D	90	51 (56.7)	50 (98.0)	1
		87	35 (40.2)	35 (100)	0
		30	20 (66.7)	20 (100)	0
	E	126	0 (0.0)	0 (0.00)	0
		94	1 (1.06)	0 (0.00)	1
		72	0 (0.00)	0 (0.00)	0

grew in all seven tested strains, and *Pca* grew in four of the five tested strains. Fluorescent *Pseudomonas* such as *P. cichorii*, *P. viridiflava*, and the *P. fluorescens* complex also grew vigorously (Table 1, Fig. S1a). *Psm* and *Pca* formed initially translucent and eventually black colonies on DTarTA in approximately 3–5 days (Fig. 1). In relation to the *Psm* strains, the ratio of the number of colonies growing on DTarTA to that on KB was 0.64 to 0.80. Regarding the *Pca* strains, the ratio of the number of colonies growing on DTarTA to that on KB was as low as 0.45–0.71 (Fig. 2). MAFF 730087 did not form colonies (Fig. 2).

Selectivity of DTarTA

Using the aforementioned five sets of seed samples, the growth-suppressing effects of DTarTA on seed bacteria were investigated. The suppression efficiency varied depending on the seed sample, and the number of colonies was reduced by 85–100% compared to that on the KB medium (Table 2). The colonies of several saprophytic bacteria were black in color and appeared similar to the colonies of *Psm* or *Pca* (Fig. S2). Seeds inoculated with *Psm* or *Pca* were mixed into these seed lots, and an attempt was made to isolate the inoculated bacteria. In seed samples B, C, and D (approximately 10^4 CFU/mL of saprophytic bacteria in the extract), 2.2–100% of the colonies grown on the medium were *Psm* or *Pca*, and it was possible to distinguish between saprophytic and inoculated bacteria based on morphological characteristics (Table 3). In seed samples A and E (approximately 10^5 CFU/mL of saprophytic bacteria in the extract), *Psm* or *Pca* comprised approximately 0–10% of the colonies grown on the medium. However, it was difficult to distinguish these colonies from those of the saprophytic bacteria based on morphological characteristics because there were too many colonies on the medium, and many of these colonies had similar morphologies to the *Psm* and *Pca*.

Growth of Psm and Pca on SPamt

The lower pH of SPamt suppressed not only the growth of seed-associated bacteria but also the growth of *Psm* and *Pca* (Fig. S3). Therefore, the pH of SPamt was set at 6.8–6.6. The pH of the medium changed slightly (±0.05 or less) after sterilization at 121 °C for 20 min. Colonies were visible two days after the application of *Psm* and *Pca* to SPamt. After 3–5 days, yellowish to coffee brown colonies were formed by *Psm*, and brown to dark brown colonies were formed by *Pca* (Fig. 1). *Pseudomonas cichorii*, *P. fuscovaginae*, *P. viridiflava*, and bacteria in the *P. syringae* complex grew on SPamt, while other bacteria did not grow or grew only slightly on the media (Table 1, Fig. S1b). The ratio of the number of bacterial colonies growing on SPamt to that on KB was 0.8–1.2 (Fig. 3). No significant differences were observed in any of the strains.

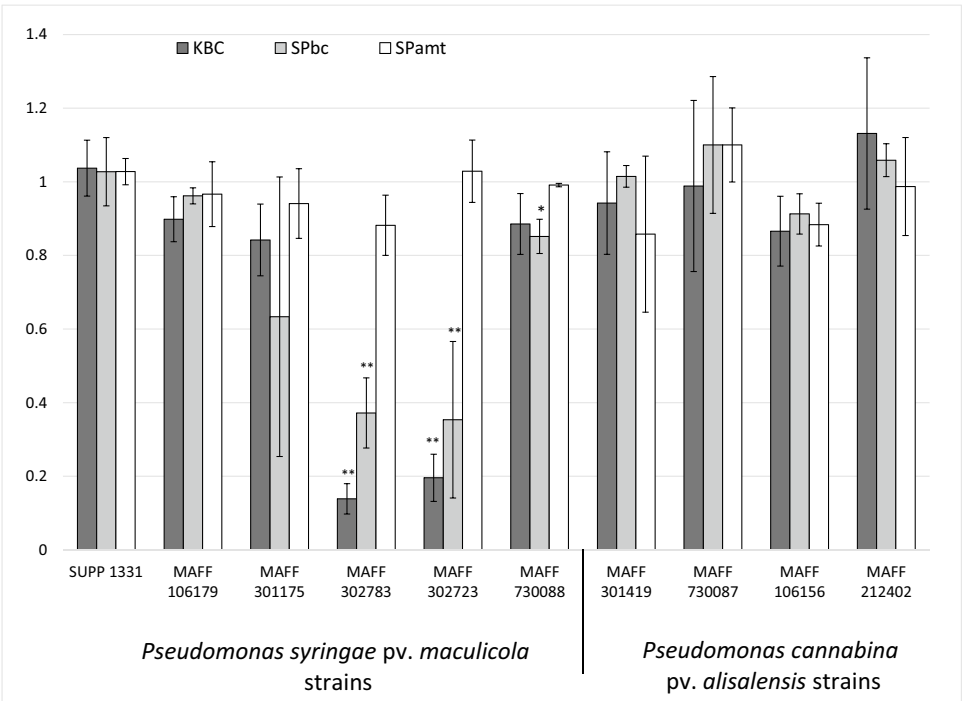

Fig. 3 Ratio of the number of colonies of *Pseudomonas syringae* pv. *maculicola* and *P. cannabina* pv. *alisalensis* on KBC, SPbc, and SPamt to that on KB. Vertical bars indicate the standard error for three independent experiments. Asterisks indicate a significant difference following Tukey's HSD test (* $P<0.05$, ** $P<0.01$)

Growth of Psm and Pca on SPbc

Psm and *Pca* formed colonies 2–3 days after plating on SPbc, and formed translucent to milky white shiny colonies at 3–5 days (Fig. 1). Bacteria in the *P. syringae* group, *P. fuscovaginae*, *P. viridiflava*, and some bacteria in the *P. fluorescens* group also grew on this medium (Table 1, Fig. S1c). The *Psm* strains MAFF 302783, 302723, and 730088 formed significantly fewer colonies on SPbc than that on KB medium (Fig. 3). Also, the plating efficiencies of MAFF 301175 on SPbc were also 45.5%, 107.0%, and 37.6% of those on KB, and the number of colonies formed was 50% or less, except in one test. For other strains, the ratio of the number of bacterial colonies growing on SPbc to the number of bacterial colonies growing on KB was 0.8–1.3.

Selectivity of SPamt and SPbc

Using the five sets of seed samples, the growth-suppressing effects of the media were investigated. The number of colonies on SPamt was 0.01–0.1 of that on KB (Table 4). The colonies of several saprophytic bacteria on SPamt were dark blue with an area of blue discoloration around them (Fig. S2). They were distinguished from those of *Psm* and *Pca* (Fig. 4) based on morphological characteristics. The colonies were confirmed to be *Psm* or *Pca* by using a growth test on YPA containing 20 mg/L of rifampicin and a specific PCR. Some bacteria formed fluid colonies and spread over the surrounding colonies. The number of colonies on SPbc was also 0.01–0.1 of that o KB (Table 4). The growth inhibitory effect of this medium was the same as that of KBC, and as the culturing period increased, the number of colonies increased significantly in some samples. The colonies of several saprophytic bacteria on SPbc were flat and pale yellow (Fig. S2) and could be distinguished from those of *Psm* and *Pca* (Fig. 4) based on morphological characteristics. The colonies were confirmed to be *Psm* or *Pca* by using a growth test on YPA containing 20 mg/L of rifampicin and a specific PCR.

Seeds inoculated with *Psm* or *Pca* were mixed into these seed sets, and an attempt was made to isolate the inoculated bacteria. In seed samples B, C, and D (approximately 10^4 CFU/mL of saprophytic bacteria in the extract), 76.5–100% of the colonies growing on the medium were *Psm* or *Pca*, and the bacteria could be identified (Table 5). In seed samples A and E (approximately 10^5 CFU/mL of saprophytic bacteria in the extract), *Pca* accounted for approximately 10–50% of the colonies growing on the media and could be distinguished from saprophytic bacteria. Among the bacteria isolated from sample A growing on SPbc, *Psm* accounted for 28.6–43.8% of the colonies growing on the medium; however, the target strains in the colonies growing in the other combinations were as low as 0–15% (Table 5). Nevertheless, it was possible to identify and isolate *Psm*.

Table 4 Reduction in the population of saprophytic bacteria isolated from different sets of *Raphanus sativus* seeds on semi-selective agar medium

	CFU of saprophytic bacteria /mL of extract											
	3 days after plating				5 days after plating				7 days after plating			
Seed set	KB	KBC	SPamt	SPbc	KB	KBC	SPamt	SPbc	KB	KBC	SPamt	SPbc
A	1.06×10^5	825 (99.2)	1.92×10^3 (98.2)	535 (99.5)	1.06×10^5	1.23×10^4 (88.2)	1.92×10^3 (98.2)	7.10×10^3 (93.3)	1.06×10^5	1.40×10^4 (86.8)	1.92×10^3 (98.2)	9.05×10^3 (91.5)
B	1.36×10^4	1.12×10^3 (91.8)	1.35×10^3 (90.1)	835 (93.9)	1.44×10^4	1.16×10^3 (91.9)	1.36×10^3 (90.6)	915 (93.6)	1.46×10^4	1.20×10^3 (91.8)	1.36×10^3 (90.7)	960 (93.4)
C	4.10×10^4	20 (99.9)	5 (99.9)	0 (100)	4.15×10^4	35 (99.9)	5 (99.9)	145 (99.7)	4.15×10^4	140 (99.7)	5 (99.9)	145 (99.7)
D	1.26×10^4	150 (98.8)	220 (98.3)	180 (98.6)	1.59×10^4	280 (98.2)	220 (98.6)	190 (98.8)	1.65×10^4	345 (97.9)	220 (98.7)	215 (98.7)
E	2.07×10^5	1.63×10^4 (92.1)	1.98×10^4 (90.4)	1.90×10^4 (90.8)	2.09×10^5	2.19×10^4 (89.5)	1.98×10^4 (90.6)	2.02×10^4 (90.4)	2.09×10^5	2.41×10^4 (88.5)	1.98×10^4 (90.6)	2.71×10^4 (87.1)

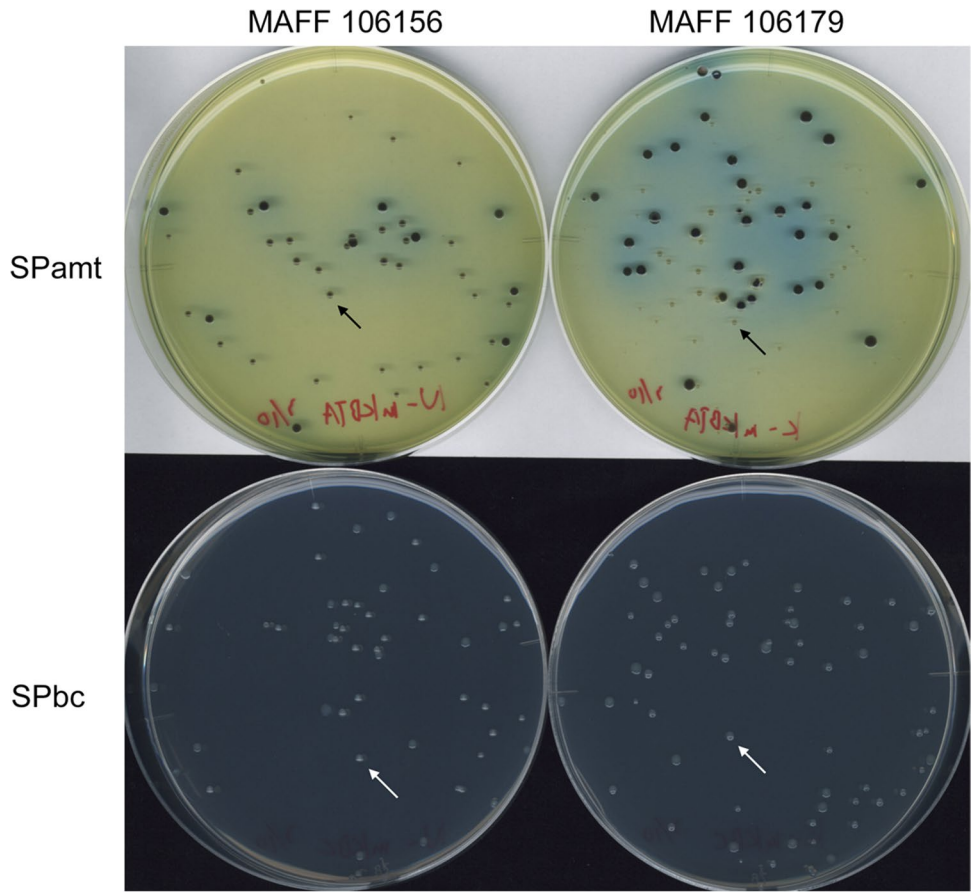

Fig. 4 Effect of *Raphanus sativus* seed-associated bacteria on the recovery of *Pseudomonas syringae* pv. *maculicola* and *P. cannabina* pv. *alisalensis* on SPamt and SPbc media. The sample contained seeds in group B (Hataro, Takii Seeds, Kyoto, Japan) mixed with MAFF 106156- or MAFF 106179-inoculated seeds. Arrows point to a typical MAFF 106156 or 106179 colony

Discussion

To date, KBC medium has been often used to isolate bacteria in the *P. syringae* group on seeds (Asaad et al. 2017; Bull and Koike 2017; Randhawa et al. 2017; Shepherd and Block 2017). However, my preliminary studies suggested that the growths of some strains of *Psm* were significantly suppressed on KBC (Fig. 2) and that it was difficult to distinguish between target and saprophytic bacteria (Fig. S2). Therefore, it was necessary to develop new selective media that suppress the growth of saprophytic bacteria and enables the formation of colonies in which *Psm* and *Pca* are morphologically different from the saprophytic bacteria.

The availability of multiple carbon sources was investigated during the development of SPTPsjA (Mori et al. 2019), and several carbon sources were evaluated in more detail in this study. Among the carbon sources evaluated, D-tartaric acid was highly selective for *Psm* and *Pca* (Table S1). Billing (1970) reported that D-tartaric acid is rarely used by any bacteria other than pv. *tomato* in the *P. syringae* group. *Psm* and pv. *tomato* are closely related (Peters et al. 2004; Wiebe and Campbell 1993), and both used D-tartaric acid and grew on DTarTA in this study. In contrast, pvs. *actinidiae* and *theae* (belonging to the same genetic group as *Psm*) and pvs. *lachrymans* and *sesami* (belonging to the same genetic group as *Pca*) did not grow in a medium containing D-tartaric acid. D-tartaric acid is rarely used in *P. cichorii* (Billing 1970), and the observed differences in the use of D-tartaric acid may be related to the host range. DTarTA medium was considered to help distinguish *Psm* and *Pca* from other strains of *P. syringae* complex.

In the DTarTA medium, it was possible to identify *Psm* or *Pca* in a sample with a bacterial density of approximately 10^4 CFU/mL in the seed extract. However, identification was difficult in a sample with a bacterial density of 10^5 CFU/mL or more, as several colonies on DTarTA were similar to those of *Psm* and *Pca*. The 16S rDNA sequences of some of the colonies on DTarTA confirmed that these were *Pseudomonas* spp. (Supplementary Information). *Pseudomonas* spp. have also been reported to survive in rapeseed and wild cabbage seeds (Granér et al. 2003; Rybakova et al. 2017; Tyc et al. 2020). In DTarTA, it was difficult to isolate *Psm* and *Pca* from seeds containing a large amount of *Pseudomonas* spp.

In this study, Spamt and SPbc media were developed. Although bacteria in the *P. syringae* complex use sucrose for energy and produce acid, several fluorescent *Pseudomonas*

Table 5 Isolation of *Pseudomonas cannabina* pv. *alisalensis* and *P. syringae* pv. *maculicola* from 5 different sets of *Raphanus sativus* seeds using semi-selective agar medium

Target bacterium	Lot of Seed	SPamt				SPbc				KBC			
		Total number of bacterial colonies	Number of target bacterial colonies	Identified as a target bacterium	Misidentified as a target bacterium	Total number of bacterial colonies	Number of target bacterial colonies	Identified as a target bacterium	Misidentified as a target bacterium	Total number of bacterial colonies	Number of target bacterial colonies	Identified as a target bacterium	Misidentified as a target bacterium
Pseudomonas cannnabina pv. *alisalensis*	A	80	40 (50.0)	40 (100)	0	12	4 (33.3)	3 (75.0)	0	80	6 (7.50)	1 (16.7)	5
	100 cfu/mL	80	31 (38.8)	23 (74.2)	1	8	3 (37.5)	2 (66.7)	0	80	5 (6.25)	5 (100)	1
		33	8 (24.2)	7 (87.5)	1	7	3 (42.9)	2 (66.7)	0	13	10 (76.9)	3 (30.0)	0
	B	43	33 (76.7)	33 (100)	0	49	34 (69.4)	26 (76.5)	0	67	42 (62.7)	36 (85.7)	14
	110 cfu/mL	52	41 (78.8)	41 (100)	0	51	36 (70.6)	35 (97.2)	0	61	44 (72.1)	43 (97.7)	0
		23	17 (73.9)	17 (100)	0	17	11 (64.7)	11 (100)	0	24	16 (66.7)	16 (100)	6
	C	14	14 (100)	14 (100)	0	20	20 (100)	20 (100)	0	8	8 (100)	8 (100)	0
	65 cfu/mL	13	13 (100)	13 (100)	0	24	23 (95.8)	23 (100)	0	12	9 (75.0)	9 (100)	2
		6	6 (100)	6 (100)	0	3	3 (100)	3 (100)	0	3	3 (100)	3 (100)	0
	D	23	15 (65.2)	15 (100)	2	24	22 (91.7)	22 (100)	0	25	24 (96.0)	23 (95.8)	0
	145 cfu/mL	25	19 (76.0)	19 (100)	0	24	17 (70.8)	17 (100)	0	22	20 (90.9)	20 (100)	0
		3	2 (66.7)	2 (100)	0	6	5 (83.3)	5 (100)	0	7	6 (85.7)	6 (100)	0
	E	59	6 (10.2)	5 (83.3)	0	65	6 (9.23)	6 (100)	0	67	4 (5.97)	3 (75.0)	3
	1360 cfu/mL	96	10 (10.4)	8 (80.0)	0	61	6 (9.83)	6 (100)	0	79	11 (13.9)	0 (0.0)	3
		33	5 (15.2)	5 (100)	0	32	3 (9.38)	3 (100)	0	38	4 (10.5)	4 (100)	0

Target bacterium	Lot of Seed	SPamt				SPbc				KBC			
		Total number of bacterial colonies	Number of target bacterial colonies	Identified as a target bacterium	Misidentified as a target bacterium	Total number of bacterial colonies	Number of target bacterial colonies	Identified as a target bacterium	Misidentified as a target bacterium	Total number of bacterial colonies	Number of target bacterial colonies	Identified as a target bacterium	Misidentified as a target bacterium
Pseudomonas syringae pv. *maculicola*	A	59	9 (15.3)	5 (55.6)	0	52	16 (30.8)	12 (75.0)	0	56	14 (25.0)	11 (78.6)	1
	100 cfu/mL	64	3 (4.69)	1 (33.3)	5	48	21 (43.8)	17 (81.0)	1	40	2 (5.00)	2 (100)	0
		24	0 (0.00)	0 (0.00)	4	14	4 (28.6)	4 (100)	0	38	3 (7.89)	2 (66.7)	0
	B	92	56 (60.9)	56 (100)	1	65	45 (69.2)	42 (93.3)	0	75	46 (61.3)	41 (89.1)	1
	305 cfu/mL	85	41 (48.2)	41 (100)	0	74	46 (62.2)	46 (100)	0	81	47 (58.0)	46 (97.9)	0
		21	9 (42.9)	9 (100)	0	13	10 (76.9)	10 (100)	0	28	18 (64.3)	18 (100)	6
	C	2	1 (50.0)	1 (100)	1	3	3 (100)	3 (100)	0	6	5 (83.3)	5 (100)	1
	80 cfu/mL	3	3 (100)	3 (100)	0	2	2 (100)	2 (100)	0	6	2 (33.3)	2 (100)	3
		1	1 (100)	1 (100)	0	3	2 (66.7)	2 (100)	0	1	1 (100)	1 (100)	0
	D	46	30 (65.2)	28 (93.3)	0	56	42 (75.0)	38 (90.5)	0	25	24 (96.0)	23 (95.8)	0
	910 cfu/mL	47	35 (74.5)	35 (100)	0	42	22 (52.4)	22 (100)	0	22	20 (90.9)	20 (100)	0
		64	41 (64.1)	41 (100)	2	67	41 (61.2)	40 (97.6)	2	7	6 (85.7)	6 (100)	0
	E	72	1 (1.39)	1 (100)	0	87	1 (1.15)	0 (0.0)	2	64	0 (0.00)	0 (0.00)	1
	80 cfu/mL	77	2 (2.60)	2 (100)	0	102	0 (0.00)	0 (0.00)	1	109	0 (0.00)	0 (0.00)	2
		84	0 (0.00)	0 (0.00)	1	69	0 (0.00)	0 (0.00)	1	88	2 (2.27)	2 (100)	0

spp. do not exhibit such metabolism (Lelliott et al. 1966). By using sucrose as the carbon source, *Psm* and *Pca* grew as rapidly as saprophytic bacteria. Peptone can be used as a nitrogen source, but the production of biosurfactants increases with the amount of peptone added (Arino et al. 1996). In SPamt, many saprophytic bacteria used peptone to raise the pH, which turned the medium and colonies blue in color. However, *Psm* and *Pca* used sucrose to produce acid, which suppressed the blue coloring. This difference in color contributed to the distinctiveness of the colonies. In SPamt, 0.5% peptone promoted blue coloring by saprophytic bacteria. In contrast, in SPbc, *Psm* and *Pca* formed milky white glossy colonies, which appeared different from the colonies of saprophytic bacteria. Therefore, BTB was not added, and the peptone content was reduced to 0.2% to suppress the growth of saprophytic bacteria.

The SPbc medium suppressed the growth of some *Psm* strains (Fig. 2), which was similar to that observed in the KBC medium. This suppression was presumed to be due to the antibiotic composition. The strains were isolated from *Brassica rapa* (Chinese cabbage and turnip) and *Raphanus* (Japanese radish). *Psm* are classified into groups I, II, and III. Group III contained many bacteria isolated from *B. rapa* and group II contained several bacteria isolated from *Raphanus* (Matsuda and Takikawa 2003; Takikawa and Takahashi 2014). Although there was no suppression of *Psm* and *Pca* growth in SPamt compared to that in KB, fluid colonies were formed in some seed extracts. The fluid colonies grew fast, making it difficult to identify and isolate *Psm* and *Pca*. No fluid colonies were formed on SPbc. Thus, both media have advantages and disadvantages in isolating these target bacteria from seeds. The combination of these media can be expected to compensate for the shortcomings of both and allow efficient isolation of these bacterial strains from seeds.

It is difficult to obtain a medium with complete selectivity for the target bacterium. In such cases, bacterial isolation would be performed using multiple semi-selective media. The seed test for black rot in cruciferous vegetables uses FS and mCS20ABN media (International Seed Testing Association 2017). Neither medium is completely selective for black rot pathogens; however, the growth characteristics of seed-borne saprophytic bacteria are different because of the various growth-inhibiting activities of these media against other bacteria. Thus, these two media are sufficient to selectively isolate black rot pathogens and allow their easy identification. For the isolation of *Psm* and *Pca* from *Raphanus* seeds, the KBTA medium was prepared using a KB medium containing the same antibiotics as those found in DTarTA (Inoue 2022). The KBTA medium was used in combination with KBC (National Agriculture and Food Research Organization 2021). KBTA promoted the growth of the Japanese radish isolates of *Psm* (Fig. S4); however, it showed poor growth of *Pca* and allowed the formation of fluid colonies. The SPamt and SPbc media developed in the current study have selectivity equal to or higher than that of KBC. Both media enhance the visibility of *Psm* and *Pca*. Based on these results, it is believed that these media can be used for the efficient identification and isolation of these bacterial strains.

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Author contribution YI conceived and designed the research, conducted the experiments, analyzed data, and wrote the manuscript.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The author declares no competing interests.

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