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Improved biovar test for *Ralstonia solanacearum*

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ABSTRACT

Race 3, biovar 2 strains of *Ralstonia solanacearum* are quarantined pathogens in Europe and Canada and Select Agent pathogens in the United States. The biovar classification of *R. solanacearum* strains is based on their biochemical abilities to utilize a carbohydrate panel. The standard biovar test uses bromothymol blue as a pH indicator in 15 ml culture tubes containing 3 to 5 ml of test media, and takes weeks to complete at 24 or 28 °C. We improved the biovar test by using phenol red as a pH indicator that changes color at a higher pH when a carbohydrate is utilized. We also conducted the test at 32 °C in 0.2 ml of 8-tube strips that reduced the medium needed by at least 20 fold. Using the improved test, biovars of *R. solanacearum* strains can be determined in 4 days when a panel of seven carbohydrates is used including glucose, trehalose, mannitol, sorbitol, dulcitol, maltose and cellobiose. To differentiate biovars 1, 2, 3 and 4, the test can be further simplified and completed in 3 days using a panel of four carbohydrates containing glucose, trehalose, maltose and dulcitol, significantly saving money, space and time.

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1. Introduction

Ralstonia solanacearum causes bacterial wilt, a soilborne vascular disease that is arguably one of the most economically important bacterial diseases in the world. It attacks over 450 plant species including ornamentals such as geranium, and limits the production of such economically important crops as tomatoes, tobacco, potatoes and bananas (Kelman et al., 1994). *R. solanacearum* is a species complex with considerable diversity. It contains many strains that differ in host range, geographical distribution, pathogenicity and biochemical properties. Strains of *R. solanacearum* are traditionally classified into 5 races based on host range (Kelman et al., 1994) and five biovars according to their ability to utilize or oxidize three disaccharides (trehalose, maltose and cellobiose) and three hexose alcohols (mannitol, sorbitol and dulcitol) (Hayward, 1964; He et al., 1983). Biovar 1 and 2 strains have a worldwide distribution, although biovar 2 strains are not known to be established in North America. Biovars 3, 4 and 5 are present mainly in Asian countries although biovar 3 strains have recently been found in Florida (Ji et al., 2007). The five races and biovars do not correspond except that in general, race 3 is equivalent to biovar 2. Race 3 biovar 2 (r3b2) causes highly destructive brown rot of potato and bacterial wilt of geranium. Compared to other strains of *R. solanacearum*, r3b2 is more adapted to cooler temperatures found in temperate climates and at higher elevations and latitudes in the tropics. *R. solanacearum* r3b2 is a quarantined pathogen in Europe and Canada, but had never been reported in the United States until 1999 when it was first introduced to the U.S. in geranium cuttings imported from Guatemala (Williamson et al., 2002). They are listed as Select Agents in the U.S. because of their potential threat to U.S. agriculture (Lambert, 2002). As a result, the accidental introductions of r3b2 into the U.S. in latently infected geranium cuttings over the years have resulted in losses of over ten million dollars to the ornamental industry and in dissolution of one major geranium company (Swanson et al., 2007). One of the best strategies to prevent further introductions and associated losses is effective exclusion through rapid, accurate and economical testing.

Biovars of *R. solanacearum* are normally differentiated using at least two independent methods, including the carbohydrate utilization-based biovar test (Hayward, 1964; He et al., 1983) and one of the DNA-based methods such as PCR or real-time PCR using specific primers (Fegan et al., 1998; Kubota et al., 2011; Thammakijjawat et al., 2006; Weller et al., 2000). Standard biovar tests (Denny and Hayward, 2001) were generally done at 24 to 28 °C in 15 ml culture tubes containing bromothymol blue as a pH indicator in 3–5 ml basal medium supplemented with a carbohydrate, and took two to four weeks to complete (Hayward, 1964; He et al., 1983; Ji et al., 2007; Williamson et al., 2002). Bromothymol blue changes color from blue to yellow when pH is lowered from 7.6 to 6.0. Phenol red, or phenolsulfonphthalein, is also a pH indicator that is commonly found and frequently used in cell biology and microbiology laboratories that changes color from pink to yellow when the pH changes from 8.2 to 6.8. As a result, it would take less time for color to

Abbreviation: r3b2, race 3 biovar 2.

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change to yellow if phenol red is used as a pH indicator. In this study, we report an improved biovar test for *R. solanacearum* that is fast, easy and economical.

2. Materials and methods

2.1. Preparation of medium for standard biovar tests

The standard biovar test medium (basal medium) (Hayward, 1964) was prepared by adding 1 g of $NH_4H_2PO_4$, 0.2 g of KCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of Difco Bacto peptone and 80 mg of bromothymol blue into a final volume of 1 l of water (Denny and Hayward, 2001). The pH was adjusted to 7.4 by drop wise addition of 5 N NaOH, in order to compare with the medium containing phenol red as described below. Glucose, trehalose, mannitol, sorbitol, dulcitol, maltose and D(+) cellobiose were filter-sterilized as 20% solutions. Dulcitol and mannitol were dissolved in water with heat. The medium was autoclaved after adding 3 g of agar. After the medium was cooled to 65 °C, 1 part of carbohydrate solution was mixed with 19 parts of the medium to obtain a final concentration of 1% of the carbohydate. Three milliliters of the mixed medium for each carbohydrate (test medium) were then dispensed into 15 ml clear culture tubes, and stored in a rack at 4 °C until use.

2.2. Preparation of improved biovar tests

The same basal medium was used in the improved test, except that phenol red was used as the pH indicator. One hundred milliliters of the medium was made by adding one milliliter each of the 100-fold stock solutions of $NH_4H_2PO_4$ (10%), KCl (2%), $MgSO_4 \cdot 7H_2O$ (2%), Difco Bacto peptone (10%) and phenol red (0.8% with the addition of one drop of 5 N NaOH in order to dissolve the dye), as well as 0.3 g of agar into 95-ml of water. One hundred and fifty microliters of the test medium was dispensed into 0.2-ml PCR 8-tube strips after 19 parts of the autoclaved basal medium was mixed with one part of a 20% carbohydrate. To ease the dispensing of the medium, the mixed medium was poured into a self-made sterilized aluminum foil boat that sat on a 25-ml ChannelMate™ reservoir insert (USA Scientific Inc., Orlando, FL), and dispensed into the tube strips using a 8-channel pipette.

2.3. R. solanacearum strains and growth

Thirty-four strains of *R. solanacearum* consisting five biovar 1, seven biovar 2, twelve biovar 3 and ten biovar 4 strains used in this study are listed in Table 1. Since biovar 5 strains were only found in China and could not be obtained, they were not included in the study. Each strain was freshly streaked from either a water or frozen stock onto triphenyltetrazolium chloride plates (Kelman, 1954) for each experiment. Inoculum of *R. solanacearum* was prepared either by suspending a couple of loopfuls of the bacterium in sterile water or by growing a single colony overnight in casamino acid peptone glucose broth (Hendrick and Sequeira, 1984) at 28 °C with shaking. Inoculum cell density was measured using OD_{600} and adjusted to 1. Twenty micro-liters of the cell suspensions were used for inoculation of the test medium in culture tubes or tube strips. Inoculum concentrations were confirmed by dilution plating. Repeat volume pipette was used to inoculate the 8-tube strips in improved biovar test. Inoculated culture tubes and tube strips were incubated at 32 °C. They

Table 1
Strains of *R. solanacearum* used in this study and their utilization of seven carbohydrates.

Biovar	Host	Strain	Origin	Source	Carbohydrate						
					Glucose[a]	Trehalose[a]	Mannitol[b]	Sorbitol[b]	Dulcitol[a]	Maltose[a]	Cellobiose[a]
1	Tomato	K60	U.S.	C. Allen, UW	+	+	−	−	−	−	−
	Tomato	Rs5	U.S.	J. Jones, UF	+	+	−	−	−	−	−
	Hydrangea	Rs124	U.S.	J. Jones, UF	+	+	−	−	−	−	−
	Geranium	Rs126	U.S.	J. Jones, UF	+	+	−	−	−	−	−
	Pond water	Rs129	U.S.	J. Jones, UF	+	+	−	−	−	−	−
2	Potato	UW224	Kenya	C. Allen, UW	+	−	−	−	−	+	+
		UW276	Mexico	C. Allen, UW	+	−	−	−	−	+	+
		UW344	Brazil	C. Allen, UW	+	−	−	−	−	+	+
		UW425	Australia	C. Allen, UW	+	−	−	−	−	+	+
		UW550	The Netherland	C. Allen, UW	+	−	−	−	−	+	+
	Geranium	UW551	Kenya	C. Allen, UW	+	−	−	−	−	+	+
		UW552	Guatemala	C. Allen, UW	+	−	−	−	−	+	+
3	Tomato	GMI1000	France	C. Allen, UW	+	+	+	+	+	+	+
		Pss4	Taiwan	This study	+	+	+	+	+	+	+
		Pss32	Taiwan	This study	+	+	+	+	+	+	+
		Pss266	Taiwan	This study	+	+	+	+	+	+	+
		Pss530	Taiwan	This study	+	+	+	+	+	+	+
	Eggplant	Pss97	Taiwan	This study	+	+	+	+	+	+	+
		Pss185	Taiwan	This study	+	+	+	+	+	+	+
	Pepper	Rs121	U.S.	J. Jones, UF	+	+	+	+	+	+	+
		Pss71	Taiwan	This study	+	+	+	+	+	+	+
		Pss106	Taiwan	This study	+	+	+	+	+	+	+
		Pss221	Taiwan	This study	+	+	+	+	+	+	+
	Potato	Pss18	Taiwan	This study	+	+	+	+	+	+	+
4	Tomato	Pss51	Taiwan	This study	+	+	+	+	+	−	−
		Pss191	Taiwan	This study	+	+	+	+	+	−	−
		Pss565	Taiwan	This study	+	+	+	+	+	−	−
		Pss901	Taiwan	This study	+	+	+	+	+	−	−
	Pepper	Pss114	Taiwan	This study	+	+	+	+	+	−	−
		Pss228	Taiwan	This study	+	+	+	+	+	−	−
		Pss267	Taiwan	This study	+	+	+	+	+	−	−
	Potato	Pss262	Taiwan	This study	+	+	+	+	+	−	−
	Sweet potato	Pss1655	Taiwan	This study	+	+	+	+	+	−	−
	Water convolvulus	Pss1283	Taiwan	This study	+	+	+	+	+	−	−

UW: University of Wisconsin, Madison, Wisconsin, USA.
UF: University of Florida, Gainesville, Florida, USA.
Complete indicator change in [a]2 to 3 days, and [b]3 to 4 days in improved biovar tests.