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Full Length Research Paper

Analysis of physiological polymorphism of Chinese Tobacco strains of *Ralstonia solanacearum*

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A collection of 41 *Ralstonia solanacearum* (RS) strains isolated from bacterial wilted tobacco collected in 10 Chinese provinces was investigated based on their biovar, heterogeneity within an axenic laboratory culture, and growth to determine the current distribution pattern of tobacco RS strains in China as well as to provide theoretic guidance to disease control. Of the 41 isolates, 18 belonged to typical biovars (bv1:1, bv2:4, bv3:10, and bv5:3) and 23 to atypical biovars. This classification was based on their ability to oxidize three hexose (lactose, maltose, and cellobiose) and three disaccharides (manitol, sobitol, and dulcite). Of the 23 strains in atypical biovars, a group closed to bv3 (utilized all the other five carbon sources except dulcite) accounted for 73.9%. Heterogeneity within an axenic culture determined through flow cytometry was initially used for RS. The results showed that the descending order rates of heterogeneity index were 19.5, 24.4, 31.7, and 24.4%, respectively. Growth of different strains in static culture showed that the rates of absorbance value from strong to weak were 58.3, 31.7, and 10%, respectively. All the results above showed that the physiological characteristics of RS strains isolated from different geographical regions were diverse.

Key words: *Ralstonia solanacearum,* polymorphism, biovar, heterogeneity, growth.

INTRODUCTION

Bacterial wilt disease caused by *Ralstonia solanacearum* (RS) is one of the most important pathogenic diseases of plants. It affects more than 450 plant species representing over 50 families distributed in the tropical, subtropical, and warm regions of the world (Wicker et al., 2007). Many economically important crops such as tomato, potato, pepper, and tobacco have been reported widely as host plants conjuring an economic loss of approximately $0.95 billion annually (DEFRA, 2003a). Bacterial wilt disease has spread northwards in the Hebei Province and southwards in the Hainan Province of China. Tobacco bacterial wilt outbreaks have recently occurred in succession on the plains of the Yangtze Valley and in the southern tobacco areas (Chen et al., 1997). In China, bacterial wilt caused by RS is a major limiting factor to the production of tobacco. Developing economical and effective control agents and breeding disease-resistant tobacco are two of the most important control measures. Effective measures to control this disease are still lacking because of its broad host range and geographical distribution, versatile infected sources, and diverse variations (Hayward, 1991; Elphinstone, 2005). Therefore, the current distribution pattern of tobacco RS strains from different geographical regions should be determined to discover a more useful counter plan against the disease, locate the origin of its occurrence, and prevent its further dissemination.

RS is recognized as a diverse species complex that differs in host range, geographical distribution,

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physiological properties, and pathogenicity. Binary classifications are used to describe its diverse intraspecific variability. Based on its host range and utilization of three hexose alcohols and three disaccharides, this pathogen is divided into five races (Buddenhagen et al., 1962; Pegg and Moffet, 1971) and six biovars (Hayward, 1964, 1991, 1994; He et al., 1983), respectively. A new phylotype classification scheme proposed by Prior and Fegan in 2005, consists of four phylotypes, each related to different geographical regions and further divided into several sequevars.

From 2007 to date, several rising molecular techniques such as multilocus sequence typing (MLST) and comparative genomic hybridization(CGH) have surfaced to understand the genetic variability of RS at a finer level of resolution (Castillo and Greenberg, 2007; Guidot et al., 2007; Darby, 2009). The polymorphism of RS could be studied using classification systems such as race, biovar and phylotype. However its diversity in other aspects (Liu et al., 2005) such as distribution in plant, morphological characteristics, and culture properties should not be neglected. Therefore, in this study, we used two new aspects (that is, heterogeneity within a population and biomass) to distinguish the polymorphism of RS in different regions. Heterogeneity or biodiversity is an index that identifies subgroups of bacterioplankton in sewage treatment systems (Andreatta et al., 2001) or micro-community in aquatic systems (Vives-Rego et al., 2000) to achieve a more detailed interpretation of community structure and ecology. Heterogeneity is generally determined through flow cytometry. Heterogeneous populations differentiated from the entire original population under axenic culture after exposure to moderate stress (e.g., antimicrobial compound, heat, UV, and so on) may also be detected through flow cytometry (Davey and Winson, 2003). Flow cytometry has several advantages (Shapiro, 1995). It can acquire several different characteristics of each cell (e.g., size, protein content, DNA content, lipid content, enzyme activity, and so on), measure at high speed (10000 cell/s to 50000 cell/s), and provide statistical mean and distribution for a given population. All these advantages have made flow cytometry a potential technique for heterogeneity studies (Shapiro, 2000; Davey and Winson, 2003). The diverse intraspecific variability of RS suggests that heterogeneity within an axenic laboratory culture of this pathogen from different geographical regions may be different. Growth determined by OD_{600} is another index that can differentiate the isolates. The color of the liquid cultures after 18 h was different when the inhibition effect of K series on RS strains from China was studied (Data not shown).

In recent years, our laboratory has made significant progress in developing new effective compounds (K series) to control RS (Wang et al., 2010; Zhao et al., 2011a, b, c, 2012). We have attained notable results after testing tobacco production areas of five provinces in China. Differentiating RS from diverse geographical areas to produce a more useful counter plan against the disease has always been a fundamental and urgent task in our laboratory. The present study mainly focused on the diversity of physiological properties (biovar, heterogeneity, and biomass) to gain a better understanding on the epidemic pattern of RS and provide theoretic guidance on disease control.

MATERIALS AND METHODS

Bacterial strains and culture

A total of 41 strains of RS collected in 2010 were isolated from typical wilted tobacco plants from 10 provinces of China: Shangdong, Hubei, Hunan, Anhui, Sichuan, Chongqing, Guizhou, Guangdong, Fujian, and Yunan. Molds on the outside were killed by washing the symptomatic plants and wiping the surface with 70% alcohol to reduce the interference of miscellaneous mircoorganism. We made a series of 1:10 dilutions of the suspension to different concentrations and palted 100 µL of the appropriate concentration (1-2 × 10^3 bacteria/mL) onto TZC agar (nutrient agar supplemented with 0.005% tetrazolium chloride) (Kelman, 1954, French, 1995). Several 5 cm long stem samples were cut from the black stem, suspended in clear water in a container, and placed on a rotary shaker at moderate speed for 20 min. The agar plates were incubated at 35°C for 48 h. The morphology of the isolates were then observed. All strains were stored as suspensions in sterile distilled water or freeze dried. For the reviving and testing, the strains were plated on TZC medium. Inoculum was prepared by adding several loopfuls of 24 to 48 h old bacterial cultures on TZC plates to 3 to 5 mL sterile distilled water to make a suspension containing approximately 1 × 10^8 bacteria/mL (OD_{600}≈0.1). The suspensions were stored at 15 to 20°C for subsequent tests (Satoshi, 1982; Denny and Hayward, 2001).

Biovar determination

Carbon source utilization

Carbon source utilization was performed following the methods described by Hayward (1964, 1994) to differentiate the biovars of Chinese tobacco RS strains. Sufficiently prepared 10% aqueous solutions of filter-sterilized glucose, lactose, maltose, cellobiose, mannitol, sorbitol, and dulcitol were added to warm basal medium ($NH_4H_2PO_4$, 1 g; KC_1, 0.2 g; $MgSO_4 \cdot 7\ H_20$, 0.2 g; peptone, 1 g; agar, 3 g; bromothymol blue, 80 mg; 1 L, pH 7.0–7.1; sterilized by autoclaving at 121°C for 20 min and cooled to 55–60°C) to produce a final concentration of 1%. After mixing, approximately 3 mL of the molten medium was dispensed into the sterilized cultured tubes (150 × 10 mm) and allowed to solidify. Freshly cultured RS cells of suspension were added to the surface of the medium in each tube and incubated at 33 to 35°C for 28 days. The color change in each tube was recorded daily. The change in the color of the medium from green to yellow indicates positive cultures. This result suggests that RS utilized the added carbohydrate and thus changed the pH of the medium. Each test was repeated three times. Non-inoculation was used as negative control, and inoculation into medium containing glucose was used as positive control.

Nitrate reduction and gas from nitrate

Using the medium of Van den Mooter et al. (1987), reliable results

on nitrate reduction and gas production (Denny and Hayward, 2001) were obtained. Into 150 × 10 mm culture tubes, 5 mL of 0.1% KNO_3 medium (KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7\ H_20$, 0.2 g; glycerol, 2 mL; KNO_3, 3 g; yeast extract, 5 g; agar,1 g; 1 L) was added. The tubes were then sterilized through autoclaving at 121°C for 20 min. Freshly cultured RS cells were stabbed into six tubes (one strain), three of which were sealed using molten 3% water agar. All tubes were incubated at 33 to 35°C for 3 to 7 days. For one week, the sealed tubes were checked for the presence of trapped gas bubbles. The unsealed tube was added with 50 µL of starch iodide solution (starch, 0.4 g; $ZnCl_2$, 2 g; distilled water 100 mL; the starch was added into warm water containing $ZnCl_2$, allowed to stand for one week, and filtered. An equal volume of 0.2% KI was added before use. Hydrochoric acid (concentrated hydrochloric acid, 16 mL; distilled water, 84 mL) was added to test the production of nitrite if the color turned blue. Non-inoculation was used as negative control.

Analysis of heterogeneity

To index the relative size and complexity of RS cells, we performed flow cytometry analyses using a FACS Calibur equipped with 15 MW 488 nm argon laser and 633 red diode laser. Every parameter (gating, relative size, and complexity) was performed using specific light sources (laser and photomultiplier) and specific detectors without fluorescent probes. Freshly cultured RS cells were stabbed into 50 mL NA broth and incubated at 35°C for 18 h. Cultures were diluted 100 times to achieve the concentration of 1×10^6–10^7 bacterium/mL. Once the instrument was adjusted properly, events were examined using the forward scatter detector (FSC; 488 nm argon laser and diode detector) and the side scatter detector (SSC; photomultiplier tube and 90° collection lens) and represented in FSC vs. SSC density plots. FSC and SSC were used as indicators of cell size and cell granularity, respectively. Acquired signals (FSC and SSC) with the amplifiers were set to liner amplification. The final setting was FSC-E00v, SSC-690v, FL1-150v, FL2-150v, and FL3-150v.

Growth determination

Growth in static culture was determined. Freshly cultured RS cells were stabbed into 50 mL NA broth and incubated at 35°C for 18 h. The optical absorbance at 600 nm of RS cultures was adjusted to approximately 0.35 with fresh NA broth under aseptic operation. A volume of 100 µL suspensions adjusted above was inoculated into 10 mL NA broth incubated at 35°C. The absorbance after 8 h incubations was determined using a spectrophotometer (722s, SHANGHAI PRECISION & SCIENTIFIC INSTRUMENT CO. LTD., Shanghai, China). Each strain was repeated three times.

Data analysis

The carbon source utilization profiles were used to measure physiological similarity among strains. Each result of carbon source utilization was scored as either 1 or 0 based on whether the tested strain can use it or not, respectively. Dendograms were constructed through the SPSS 12.0 software using the unweighted pair-group method with arithmetic means algorithm (UPGMA). Data collected by FCM were presented in FSC vs. SSC density plots using the WinMDI 2.9 software. Excel 2003 was used to calculate the index of heterogeneity, and K-means method was performed to divide the RS isolates into different clusters. Growth in static culture was presented using GraphPad Prism, which directly mapped the value of OD_{600} clustered using the K-means method.

RESULTS AND ANALYSIS

Isolation of RS

A total of 41 strains of RS (Table 1) collected in 2010 were isolated from typical wilted tobacco stems from 10 provinces of China: Shangdong, Hubei, Hunan, Anhui, Sichuan, Chongqing, Guizhou, Guangdong, Fujian, and Yunan. All were numbered as “Tbxy10n” (“Tb” stands for Tobacco, “xy” stands for the initials of the province, “10” denotes the year 2010, and “n” is the code of the individual isolated strain). The typical colony morphology of the isolates on TZC medium is shown in Figure 1. Most of them produced a normal round pink pigment colony with a slimy and milky outer ring on TZC medium.

Biovar assignment

Of the 41 isolates, 18 belonged to typical biovars (bv1:1, bv2:4, bv3:10, and bv5:3) and 23 to atypical biovars. This classification was based on their ability to oxidize three hexose (lactose, maltose, and cellobiose) and three disaccharides (manitol, sobitol, and dulcite) (Figure 1). Each result of carbon source utilization was scored as either "1" or "0" based on whether the tested strain can use it or not, respectively. A binary matrix was then obtained. A dendrogram was constructed using the statistical software SPSS 12.0 using the UPGMA algorithm (Figure 2). Of these typical bivars, bv 3 was accounted for 55.6% and obtained from Guizhou province, except Tbcq10-2, isolated from Chongqing. None of the isolates was assigned to biovar 4. Of the 23 strains in atypical biovars, a group close to bv3 (utilized all the other five carbon sources, except dulcite) accounted for 73.9%. Figure 2 shows the three major types of tobacco RS in China. The first type contained bv3 and a group close to bv3, which accounted for 65.7% of the 41 isolates. The second type consisted of bv2 and bv3, which can only use three or four carbohydrates and accounted for 19.5%. The third type consisted of strains that can only utilize less than two carbon substances and accounted for 14.7%. Therefore, the first type is the majority. Of the 41 strains, 26 isolates were obtained from Guizhou province. All typical and atypical biovars were presented in Guizhou, and the major biovars were still congruent with those in China.

Heterogeneity within RS species

Although parameters such as FSC and SSC can reflect the size and complexity of cells relatively, the absolute value remains an arbitrary unit. These parameters are difficult to compare between strains without an absolute scale. Our laboratory initially restructured these relative indices and determined heterogeneity within an axenic culture in an absolute scale. The FSC vs. SSC density

Table 1. Strains of *Ralstonia solanacearum* used in this study.

Code no.	Strains	Geographic origin	Biovar	Gas	H	D	8h-biomass(OD_{600})
1	Tbgz10-1	Yinjiang, Guizhou	Atypical	+	2.352Hb	0.728	0.286±0.026[a]
2	Tbgz10-2	Yinjiang, Guizhou	5	+	2.469Ha	0.754	0.236±0.006[a]
3	Tbgz10-3	Jiangkou, Guizhou	5	+	2.682Ha	0.798	0.289±0.003[a]
4	Tbgz10-4	Dejiang, Guizhou	5	+	1.766Hd	0.642	0.300±0.016[a]
5	Tbgz10-5	Sinan, Guizhou	2	-	1.877Hc	0.672	0.376±0.022[b]
6	Tbgz10-6	Shiqian, Guizhou	3	+	1.713Hd	0.552	0.580±0.011[c]
7	Tbgz10-7	Shiqian, Guizhou	3	-	1.986Hc	0.669	0.550±0.006[c]
8	Tbgz10-8	Suiyang, Guizhou	3	+	2.561Ha	0.785	0.447±0.000[b]
9	Tbgz10-9	Tongzi, Guizhou	Atypical	+	1.813Hd	0.681	0.567±0.011[c]
10	Tbgz10-10	Zuiyi, Guizhou	1	+	1.957Hc	0.675	0.201±0.007[a]
11	Tbgz10-11	Daozhen, Guizhou	2	+	1.699Hd	0.610	0.246±0.012[a]
12	Tbgz10-12	Yuqing, Guizhou	Atypical	+	2.273Hb	0.734	0.267±0.010[a]
13	Tbgz10-13	Qianxi, Guizhou	3	+	1.892Hc	0.691	0.582±0.021[c]
14	Tbgz10-14	Dafang, Guizhou	Atypical	-	1.501Hd	0.589	0.500±0.012[b]
15	Tbgz10-15	Bijie, Guizhou	3	+	1.942Hc	0.690	0.551±0.026[c]
16	Tbgz10-16	Jinsha, Guizhou	3	+	2.398Ha	0.763	0.500±0.011[b]
17	Tbgz10-17	Huishui, Guizhou	3	+	1.982Hc	0.673	0.514±0.016[c]
18	Tbgz10-18	Fuquan, Guizhou	Atypical	+	2.050Hc	0.697	0.119±0.005[d]
19	Tbgz10-19	Weng'an, Guizhou	2	+	1.863Hc	0.659	0.389±0.005[b]
20	Tbgz10-20	Weng'an, Guizhou	Atypical	+	1.745Hd	0.658	0.556±0.006[c]
21	Tbgz10-21	Fuquan, Guizhou	3	+	2.435Ha	0.745	0.063±0.006[d]
22	Tbgz10-22	Pingtang, Guizhou	Atypical	-	1.771Hd	0.631	0.480±0.013[b]
23	Tbgz10-23	Dushan, Guizhou	Atypical	+	1.984Hc	0.681	0.240±0.008[a]
24	Tbgz10-24	Majiang, Guizhou	Atypical	+	2.237Hb	0.695	0.273±0.014[a]
25	Tbgz10-25	Tianzhu, Guihou	Atypical	-	2.481Ha	0.765	0.408±0.011[b]
26	Tbgz10-26	Sanhui, Guizhou	3	+	2.426Ha	0.739	0.581±0.024[c]
27	Tbhb10-1	Xuanen, Hubei	Atypical	+	2.329Hb	0.725	0.112±0.004[a]
28	Tbhb10-2	Xianfeng, Hubei	Atypical	+	1.968Hc	0.650	0.271±0.010[a]
29	Tbyn10-1	Qiubei, Yunan	Atypical	+	1.662Hd	0.630	0.442±0.012[b]
30	Tbgd10-1	Nanxiong, Guangdong	Atypical	-	2.033Hc	0.675	0.495±0.007[c]
31	Tbhn10-1	Yongzhou, Hunan	Atypical	+	1.849Hc	0.641	0.587±0.015[c]
32	Tbsc10-1	Miyi, Sichuan	2	-	2.162Hb	0.725	0.053±0.003[d]
33	Tbsc10-2	Dechang, Sichuan	Atypical	+	1.742Hd	0.621	0.517±0.017[c]
34	Rs71	TRIA	Atypical	+	2.627Ha	0.800	0.544±0.005[c]
35	Tbah10-1	Shitai, Anhui	Atypical	-	1.770Hd	0.662	0.559±0.032[c]
36	Tbsd10-1	Laoshan, Shandong	Atypical	+	2.129Hb	0.711	0.331±0.006[a]
37	Tbsd10-2	Juxian, Shandong	Atypical	-	2.073Hb	0.709	0.309±0.018[a]
38	Tbsd10-3	Zhucheng, Shandong	Atypical	+	2.092Hb	0.686	0.281±0.004[a]
39	Tbfj10-1	Sanming, Fujian	Atypical	-	1.936Hc	0.678	0.562±0.009[c]
40	Tbcq10-1	Wangzhou, Chongqing	Atypical	+	2.183Hb	0.731	0.651±0.089[c]
41	Tbcq10-2	Qianjiang, Chongqing	3	-	2.244Hb	0.733	0.614±0.011[c]

[1] TRIA= Tobacco Research Institute of Anhui Province. [2] + indicates a positive result; - stands for a negative result; in each column followed with different lowercase letters (a, b, c, and d) indicate that they belong to different clusters using the K-means method; OD_{600} = mean±standard deviation.

plot of code no. 25 is shown in Figure 3a. To minimize electronic noise and cell fragments, gating in WinMDI was used, which is similar to R1 in Figure 3b. The bacteria in the middle of the data space (512 × 768) were partitioned into 14 small squares (R2-R15) using the "regions tool" in WinMDI (Figure 3c). Finer division should be performed in a denser zone. Each square (R2-R15) represented a subpopulation with a similar FSC and SSC in the same zone of the isolates. The rate of each subpopulation (P_i) in the whole population could be

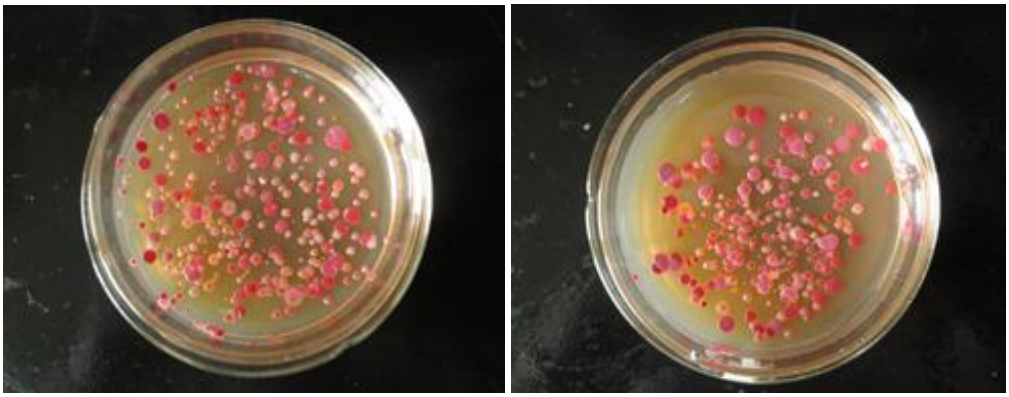

Figure 1. The colony morphology of the isolates on TZC medium after 48 h.

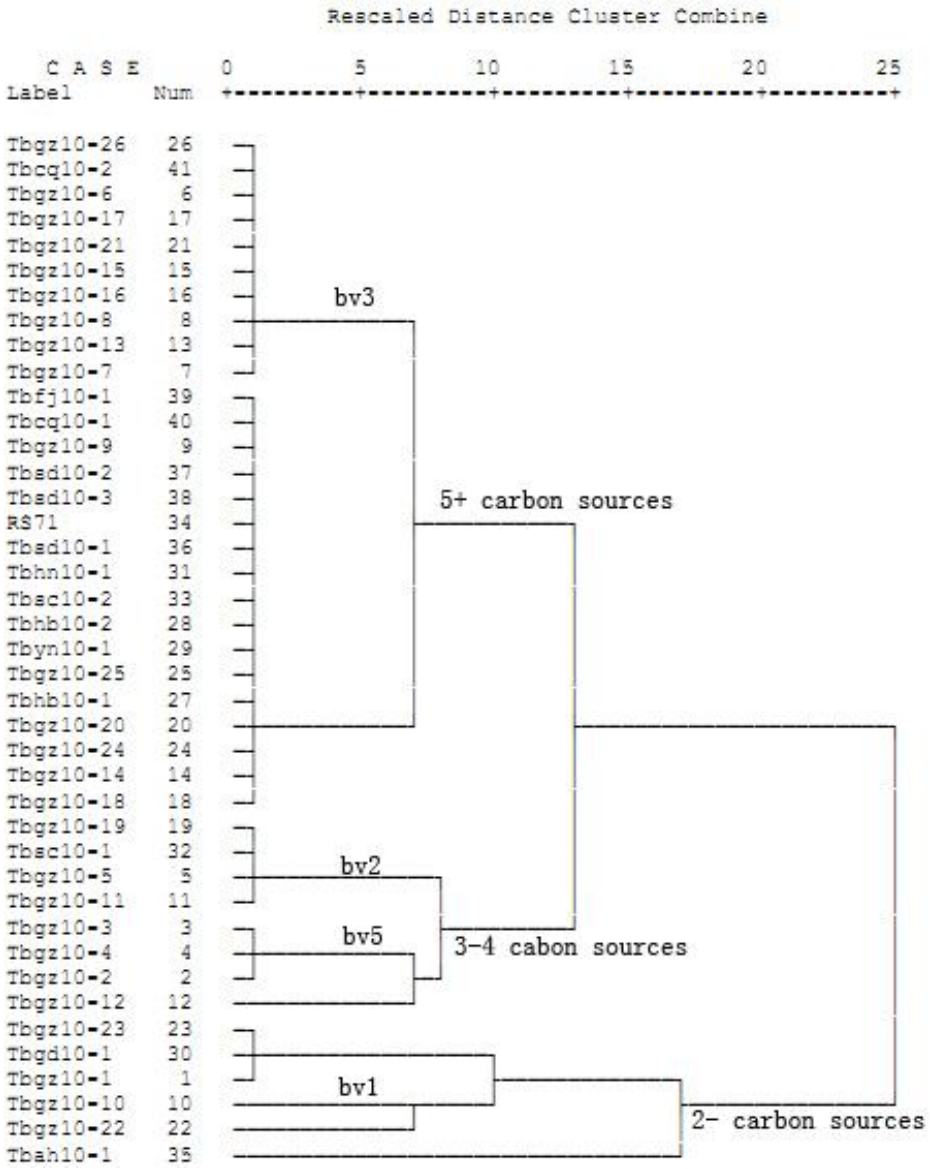

Figure 2. Biovar diversity of 41 strains of *Ralstonia solanacearum* isolated from tobacco in China.

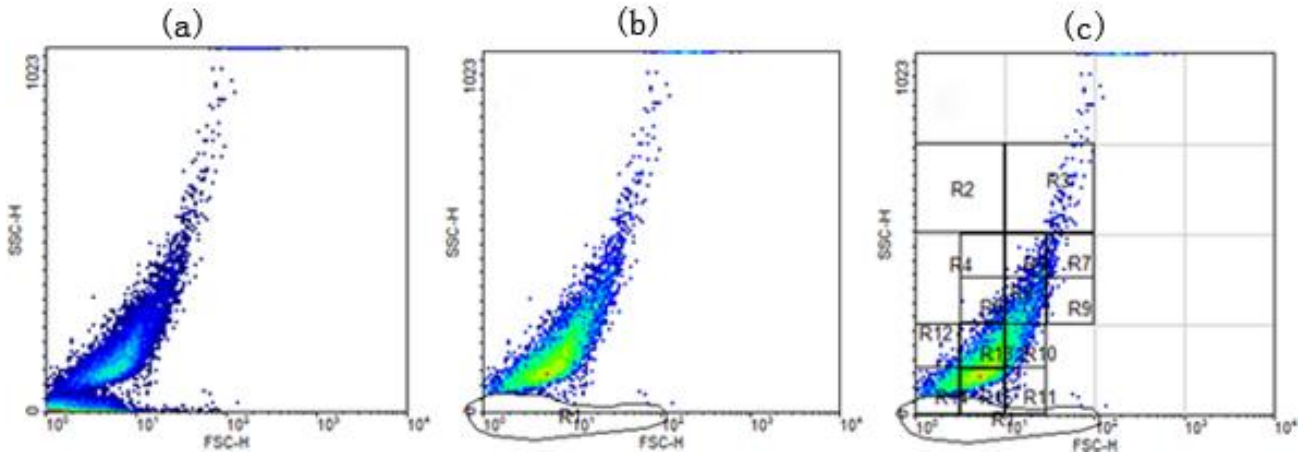

Figure 3. FSC vs. SSC density plots of the code no. 25 strain of *Ralstonia solanacearum.*

calculated using "stats" in

$$H = -\sum_{i=1}^{s} P_i \log_2 P_i$$

$$D = 1 - \sum_{i=1}^{s} P_i^2$$

WinMDI. Borrowing biodiversity index from applied ecology, such as the Shannon-Weiner Index (H) and Simpson's diversity Index (D), heterogeneity index can be obtained to characterize the physiological differentiation of cells within an axenic laboratory culture.

The axenic culture in this study was under static culture for 18 h at 35 °C during which most cells were intact (Nielsen et al., 2009). Therefore, FSC vs. SSC density plots showed the real size and complexity of cells, and heterogeneity index (H or D) presented the size and complexity differentiation of cells in RS cultures. According to the H and D formulas above, the calculated index of each strain is shown in Table 1. The K-means method was performed after constructing a binary matrix from H and D to divide RS isolates into four different clusters denoted by Ha, Hb, Hc, and Hd (Table 1). The heterogeneity index H was more evident in polymorphism than D. The exact results of H are shown in Figure 5. To prove the validity of this method, representatives (code no. 34, 32, 17, and 14) from each cluster are presented in Figure 4a–d. Each H value of the representatives was found to be consistent with the distribution of the density plots. Therefore, the size differentiation of cells may be presented by using the heterogeneity index and its calculation method developed in this study.

Figure 5 shows that heterogeneity within RS species from different regions was diversified. Moreover, the descending order rates of the heterogeneity index in China were 19.5, 24.4, 31.7, and 24.4%, respectively. Of the 26 isolates collected in Guizhou, types of H_{max} (Ha) and H_{min} (Hd) were the majority in the four different types of heterogeneity. The descending order rates of heterogeneity index in Guizhou were 26.9% (Ha), 11.6% (Hb), 34.6% (Hc), and 26.9% (Hd), respectively. Types of H_{med} (Hb and Hc) were the majority in other provinces and accounted for 73.3%, except the isolates in Guizhou.

Growth in static culture

The control chart of OD_{600} of inoculation source is shown in Figure 6. The optical absorbance was between 0.33 and 0.38, and its mean ± standard deviation was 0.354 ± 0.008. From the growth curves (static culture under 35°C) of strains Tbgz10-25 (code no. 25) and Tbah10-1 (code no. 35), the time 0 to 8 h was the exponential phase (very short lag phase), and a turning point occurred after 8 to 10 h (Data not shown). Therefore, biomass of 8 h was selected as a unified scale to compare the growth of the 41 strains (Table 1 and Figure 7). The results in Figure 7 show that the growth characteristics of RS strains isolated from different geographical regions were diverse. In addition, and the rates of absorbance value from strong to weak were 58.3% (b and c), 31.7% (a), and 10% (d), respectively. The growth characteristics of isolates from Guizhou were also polymorphic, and the rates of absorbance value from strong to weak were 26.9% (b), 30.8% (c), 34.6% (a), and 7.7% (d), respectively.

DISCUSSION

Studies on the distribution pattern of RS pathogen in China had been proceeding since 1980 based on the traditional biovar classification scheme, showing that most of the RS belonged to bv3, bv4, and bv5 (Lu, 1998; Xu and Pan et al., 2009; Xue and Yin et al., 2011), and

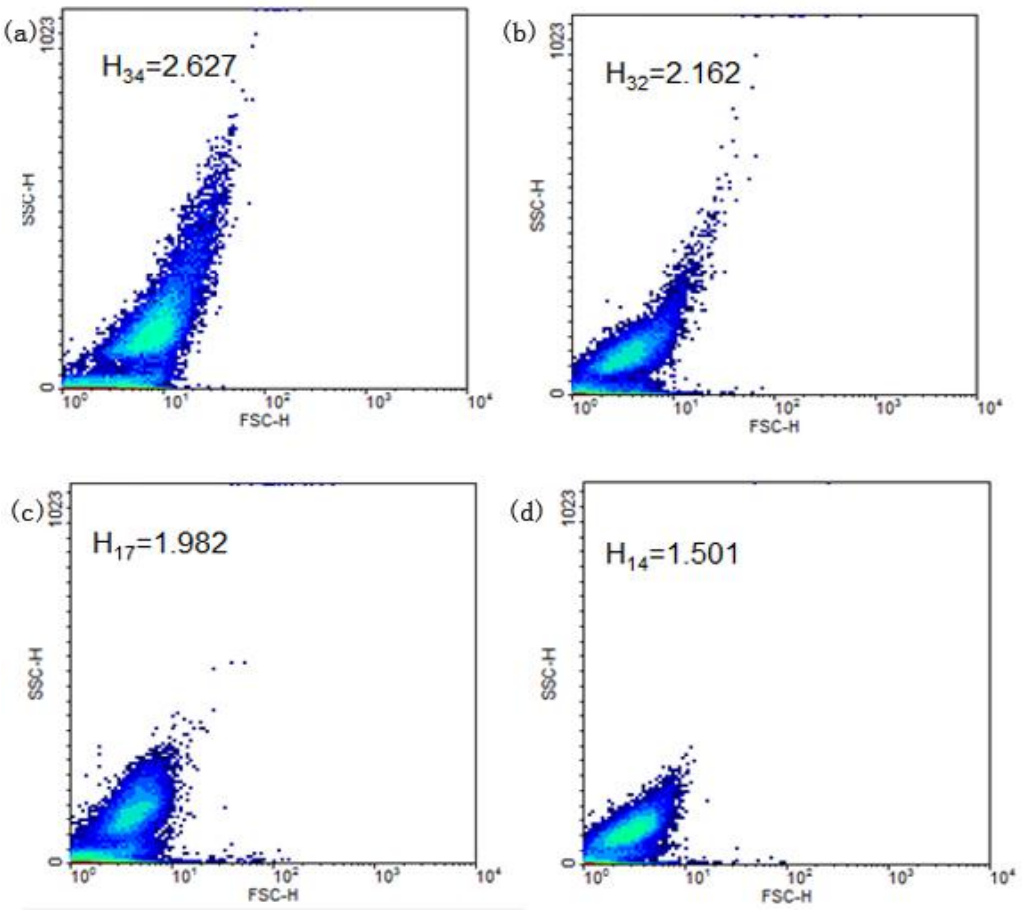

Figure 4. FSC vs. SSC density plots of representatives (code no. 34, 32, 17 and 14) from each cluster (Ha, Hb, Hc, Hd) of *Ralstonia solanacearum*.

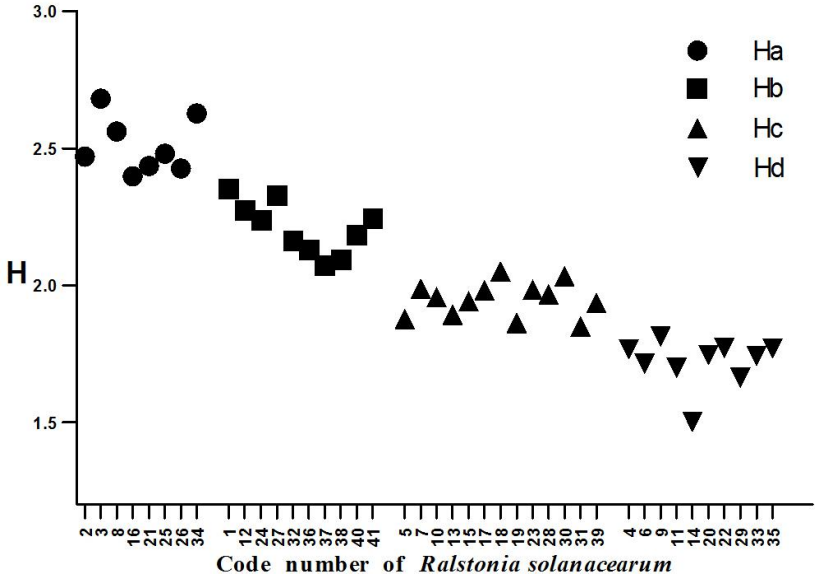

Figure 5. Heterogeneity Index of *Ralstonia solanacearum* isolated in China after 18 h growth. H1-H4 indicates they belonged to different clusters using the K-means method.

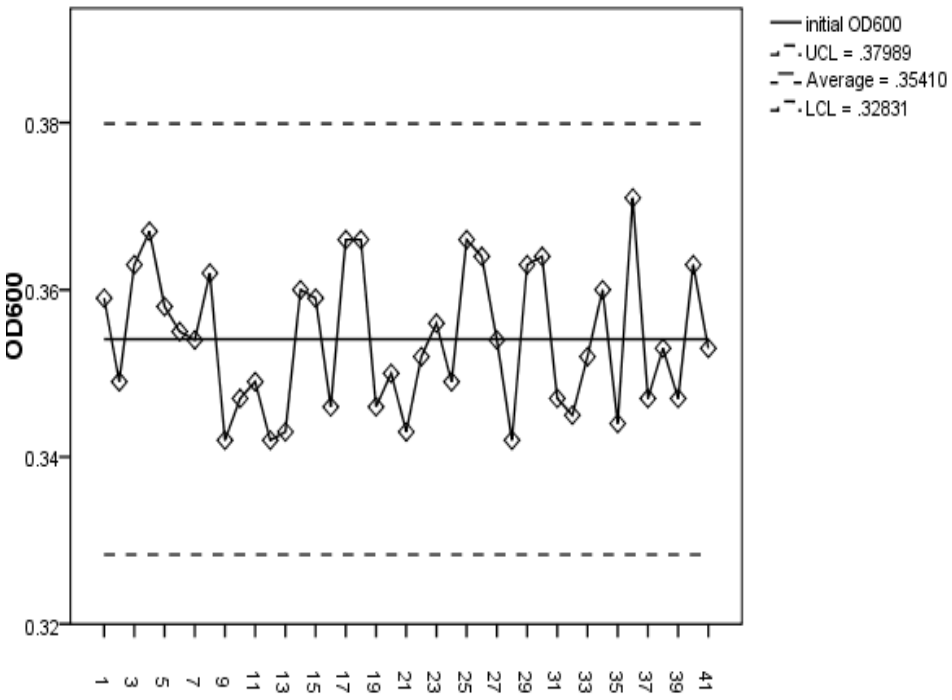

Figure 6. The control chart of OD_{600} of inoculation source of *Ralstonia solanacearum.*

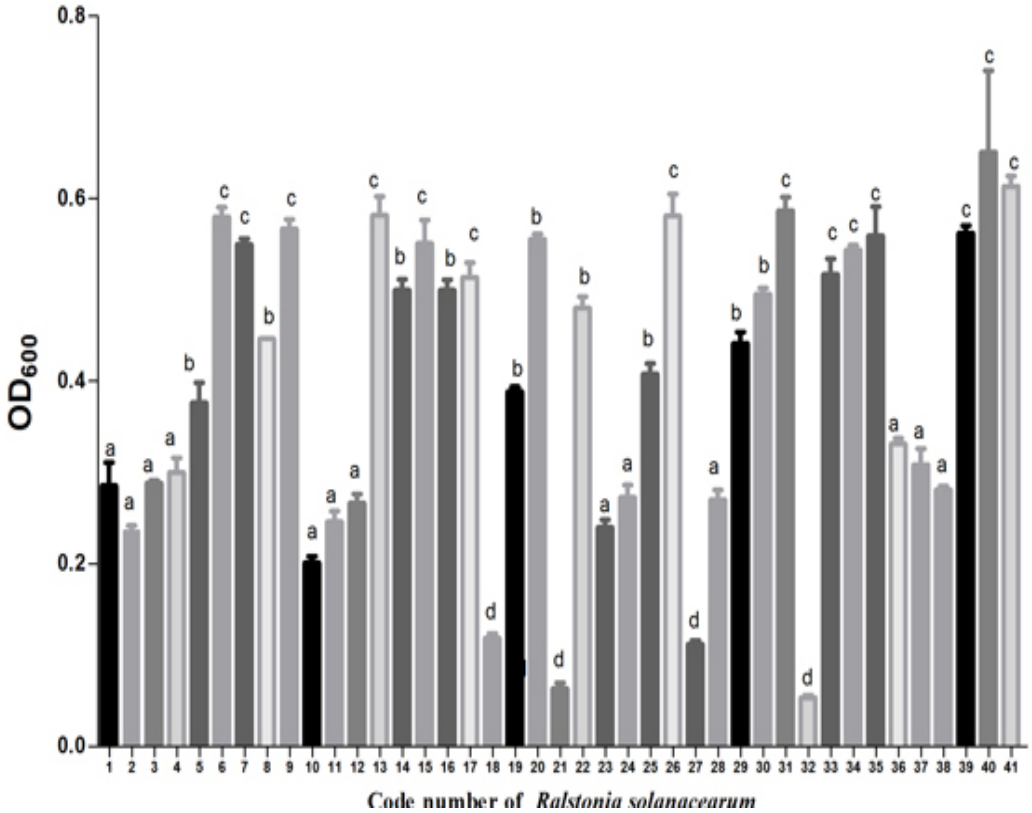

Figure 7. Growth in static culture of *Ralstonia solanacearum* at 35°C for 8 h. The bars represent standard errors. Values with different letters above the bar belonged to different clusters using the K-means method.

the vast majority of tobacco RS were assigned to bv3 and bv4 (Zheng et al., 2007; Zou et al., 2007; Wang et al., 1996). Our results are consistent with the previous reports for the majority contained bv3 and a group close to bv3. According to the report of Xu et al. (2010) on the biovar determination of 45 RS strains isolated from Fujian province, one strain belonged to atypical biovar, whereas many isolates belonged to several atypical biovars in this study. Therefore, RS strains were more diverse, based on the biovar classification scheme used in this report. None of the isolates was assigned to bv 4. Bv1 (Tbgz10-10) in tobacco RS was first reported in the present study. No scholar has reported on the economic loss caused by strain bv1 in tobacco field in China since Ji et al. (2009) isolated one strain assigned to bv1 from ginger. The pathogenicity of this newly isolated bv1 strain (Tbgz10-10) will be tested further.

Size differentiation of cells using the heterogeneity index determined through flow cytometry was first used for RS. The calculation method developed on H value showed that they were consistent with the distribution of the density plots. Generally, heterogeneity (biodiversity) is an index that characterizes community structure and evaluates the stability in macroscopic ecological system. In terms of microscopic ecological systems, especially micro-communities in deep sea or Antarctic and Arctic aquatic systems, special and powerful supplementary means are needed. Flow cytometry is a potential and useful technique to achieve these goals. The method developed on the heterogeneity index of microcosm through flow cytometry and the “region” tool of winMDI not only can be applied in axenic cultures but also in several other areas such as morphology change in mitochondrial subpopulations caused by diabetic cardiomypathy-associated dysfunction (Dabkowski et al., 2008, 2009). The absolute size change in mitochondrial subpopulations cannot be determined. However, the morphology change performed by heterogeneity index can determine which subpopulation is more impacted while saving a large fund to buy reagents.

Studies on the growth curve of microorganism generally include continually renewing nutrients, regulating temperature, and illumination to improve production in fermentation engineering. The growth of microorganisms changes when conditions such as nutrients, temperature, and illumination change. However, the growth differentiation of intraspecific strains depends on their own properties and is not related with the unified conditions controlled in culture. Horiata et al. (2005) once used the OD_{660} of RS growth in static culture for several hours to show the biochemical differences between Asian and South American stains of bvN2 and bv2. Their results showed that the average absorbance value of the Asian strains was significantly lower than that of the South American strains at 27 and 32°C for 48 h. In addition, they revealed that the OD_{660} of bv2 strains was drastically lower than bvN2 strains from Asian and South American at 27, 32, and 37°C. The growth of 41 tobacco strains in this study was significantly diverse, which may be the consequence related to the interaction of versatile genes and diversified climates. Further study needs to be conducted on determining how they interact to make growth polymorphic.

The present study aims to determine the current distribution pattern of tobacco RS strains from different geographical regions. The results of this study may help locate the origin of their occurrence and pointedly find more effective anti-bacteria agents against the disease. Four different groups were found when the inhibition effect of 50 mg/mL K1, 50 mg/mL K2, 50 mg/mL K3, and 50 mg/mL Agri-streptomycin were compared using the UPGMA algorithm (data not shown). The correlation between the physiological polymorphism and the selective inhibition effects of anti-bacterial agent K series and agricultural streptomycin showed that the physiological characteristics (biovar, heterogeneity, and growth) of strains "only unsusceptible to K1" (Tbgz10-1, Tbsc10-2, Tbyn10-1, Tbfj10-1, and Tbgz10-25) and strains "only susceptible to K1" (Tbgz10-16, Tbgz10-24, Tbgz10-7, and Tbgz10-1) were specifically different. One group contained isolates Tbgz10-12 and Tbcq10-1 whose biochemical properties were different from the rest of the isolates (data not shown). To interpret the selective inhibition effects of different anti-bacterial agents on RS strains based only on physiological polymorphism (phenotype diversity), several drawbacks in the postgenomic era and virulence factors of some reference strains have been studied in great detail (Darby, 2009). The complete genome of RS GM1000 has been sequenced (Guidot et al., 2007). An analysis of five housekeeping genes and three virulence-related genes of 58 RS strains from worldwide suggested that geographic isolation and spatial distance play a role in evolution (Castillo and Greeenberg, 2007). Studies on phenotype diversity and genotype diversity should be merged. More powerful DNA-based methods such as phylotype classification scheme, MLST, and BOX, are needed to reveal epidemiolopgy and provide theoretic guidance for disease control.

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