TAIWANIA, 54(1): 37-44, 2009

Effects of Stress Factors on the Multiplication and Survival of a Taiwan *Ralstonia solanacearum* Tomato Strain

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(Manuscript received 25 August 2008; accepted 9 November 2008)

ABSTRACT: Microbes encounter various environmental factors which frequently cause detrimental effects on their population dynamics. *Ralstonia solanacearum* is a devastating bacterium causing a deadly and very complex disease of many agronomically important crops. To better control the disease, information about responses of distinct strains of this bacterium to environmental factors is certainly important but still insufficient. In this study, effects of salinity, excess copper, extreme pHs, drought and light on the propagation and survival of a representative of *R. solanacearum* strains in Taiwan, Pss4, were assessed. Our results showed that salinity negatively affected Pss4 multiplication in rich media at a dose-dependent manner, but resulted in negligent effects on its survival in sand and in water. In the presence of excess copper, similar negative effects on Pss4 culturability in rich media and at a less level on that in sand occurred. Additionally, Pss4 multiplication in rich media was substantially reduced at pH 9.0 and at a less degree at pH 5.0. Moreover, the survival of this bacterium in sand was sensitive to drought, but it sustained under incident light in water. The results were compared with previous studies on distinct *R. solanacearum* strains prevalent in different geographic regions and discussed in detail. The information gathered from this study would pave to the way for future studies on field samples to advance disease epidemiology and develop feasible strategies for disease control in Taiwan.

KEY WORDS: *Ralstonia solanacearum*, multiplication, survival, environmental factors.

INTRODUCTION

Bacterial wilt is a very complex and deadly soil-borne vascular disease of many agronomically important crop species (Jaunet and Wang, 1999; Wang et al., 2000). The disease occurs mainly in tropic, sub-tropic and warm temperature zones, but now has extended to more temperate areas (Kim et al., 2003). Production loss due to this disease can be 100% as conventional control strategies for this disease are not effective. The causing agent of bacterial wilt, *Ralstonia solanacearum*, is a gram-negative, aerobic rod bacterium belonging to β-proteobacteria. This bacterium has an unusual wide host range covering over 200 species and can survive in soil for a long period of time (Hayward, 1991). Based on biochemical, molecular, and metabolic characteristics, *R. solanacearum* could be classified into four phylotypes, five biovars, five races and many strains (Denny, 2006; Castillo and Greenberg, 2007). The strains in race 1 are highly diverse both in their genotypes and aggressiveness (Jaunet and Wang, 1999). Due to the diversity of this bacterium, variations in stability of host plant resistance over different field locations exist (Lopes et al., 1994; Hanson et al., 1998).

In the natural infection process, *R. solanacearum* invades plants at the sites of emergence of secondary roots or at root tips, propagates intercellularly and then enters into the xylem system (Vasse et al., 1995). The infection then becomes systemically, with further bacterial multiplication and the production of large amounts of extracellular polysaccharides, leading to complete wilting and the death of infected plants (Buddenhagen and Kelman, 1964). The bacterium then returns to the soil, where it can be associated with plant debris and weed rhizosphere and survive under humid conditions over a long time. Previous reports suggested that *R. solanacearum* may develop a viable-but-nonculturable state, which could be involved in long-term survival of the bacterium and in plant infection (Grey and Steck, 2001).

Being a very "environmentally conscious" microorganism capable of actively multiplying under nutrient-adequate conditions and surviving in natural environments, population dynamics of *R. solanacearum* is strongly influenced by various environmental factors. For instances, highly differential effects of amendment regimes on the survival of *R. solanacearum* occurred in different soil types from various regions (Michel and Mew, 1998; Messiha et al., 2007). In Dutch irrigation water, seawater salts, soil drying, extreme temperatures, incident light and microbiota have been reported to cause negative effects on population dynamics of a *R. solanacearum* biovar 2 strain (van Elsas et al., 2001). Recently, influence of extreme temperatures and native microbiota on the survival of a *R. solanacearum* biovar 2 in Spain rivers has been studied (Caruso et al., 2005;

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Àlvarez et al., 2007). Furthermore, low temperature-induced viable-but-nonculturable state of *R. solanacearum* was reported to affect the bacterial virulence (van Overbeek et al., 2004). Due to the complexity and diverse properties among *R. solanacearum* strains, gaining insights into the nature of local strains in response to environmental factors is certainly important and thus highly desired for developing feasible strategies for disease control (Schuerger and Brown, 1997; Gorissen et al., 2004; Norman et al., 2006).

The objective of this work is to set up pilot studies to evaluate effects of environmental factors on the propagation and survival of a representative local *R. solanacearum* strain, Pss4. Pss4 was isolated from tomato plants, belongs to race 1 and biovar 3, and possesses a typical level of aggressiveness commonly conferred in strains prevail in Taiwan fields (Jaunet and Wang, 1999). Important environmental factors, including salinity, excess copper, extreme pHs, drought and light, were tested to assess their influence on the multiplication and survival strain of Pss4 under experimental conditions. The results were further compared with all available previous studies carried out on distinct *R. solanacearum* strains prevalent in other geographic regions.

MATERIALS AND METHODS

Preparation of *R. solanacearum* inocula

R. solanacearum Pss4 isolated from tomato plant in Taiwan was used for this study. The bacterium was grown in 523 broth (0.03% magnesium sulfate heptahydrate, 0.2% potassium phosphate, 0.4% yeast extract, 0.8% casein hydrolysate, 1% sucrose, and/or 1.5% Bacto agar) for 2 days at 28℃. Bacterial cells were harvested by centrifugation (5000 ×g, 10 min) and washed twice in autoclaved Milli-Q filtered water. Bacterial suspensions at an OD_{600} equal to 0.3 (about 10^8 CFU/mL) were prepared for stress response tests.

Stress tests in liquid media

For salinity and copper stress tests, 523 liquid media containing different concentrations of NaCl (0~0.2 M) and $CuSO_4$ (0~4 mM) were prepared. For extreme pH tests, 523 liquid media were prepared to give various initial pHs (5.0 to 9.0) with NaOH or HCl. Prior to inoculation into the media, a bacterium suspension at an OD_{600} equal to 0.3 was diluted 10 folds in 523 medium, and 0.5 mL of the diluted bacterium suspension was added into each of 100-mL flasks containing 25 mL of test media. The bacterial cultures were incubated at 28°C in dark with constant shaking at 200 rpm. Bacterial titers were determined over time by serial dilutions and plating on modified SM1 medium (1% peptone, 0.1% casein hydrolysate, 0.5% glucose, 1.5% Bacto agar, 0.5% TTC, 0.01% polymyxin B Sulfate, 0.002% tyrothricin, 0.005% chloramphenicol, 0.005% cycloheximide, and 0.005% crystal violet). Data collected from at least three independent trials were analyzed.

Stress tests in solid media

For monitoring bacterial colony formation, 523 solid media were prepared in the presence of the respective stress factors as described above. A bacterium suspension at an OD_{600} equal to 0.3 was diluted in 523 medium to give a concentration of 10^2 CFU/mL. Two hundred microliters of the diluted bacterium suspension was plated on each of the test plates and incubated at 28°C in dark. Two plates were prepared for each test and the size of bacterial colonies was monitored over time. Data collected from at least three independent trials were analyzed.

Stress tests in sand

Autoclaved black sand was used for these tests. Solutions of NaCl (0~0.1 M) and $CuSO_4$ (0~8 mM) were prepared in autoclaved water. A bacterium suspension at an OD_{600} equal to 0.3 was diluted 10 folds in autoclaved water. For each sample, 50 grams of sand was first mixed well with 5 mL of the diluted bacterium suspension. For drought stress tests, the samples were kept in 250-mL dishes and incubated at room temperature in a confined dark space, with or without adding 20 mL of autoclaved water weekly. For salinity and copper stress tests, the bacterium- inoculated sand was mixed thoroughly with 20 mL of treatment solution at different concentrations, placed in polyethylene bags, and incubated at 28°C in dark. Each week during the experiments, 5 grams of sand was taken from the samples, mixing with 200 mL of autoclaved water, and stirred at room temperature for 30 min. Bacterial titers were then determined by serial dilutions and plating on modified SM1 medium. Data collected from at least three independent trials were analyzed.

Stress tests in water

Flasks (100-mL) filled with 25 mL of autoclaved MilliQ-filtered water (pH 8.3) or NaCl solutions of different concentrations (0~0.2 M) were prepared, and 0.5 mL of the bacterium suspension (diluted 10 folds in autoclaved water from an OD_{600} equal to 0.3) was added into each of the flasks. The bacterial cultures were incubated at 28°C with constant shaking at 200 rpm in dark for NaCl stress tests or under a 16/8 h day/night photoperiod in a plant growth chamber with a light intensity at 5 W/m^2 for incident light tests. Bacterial

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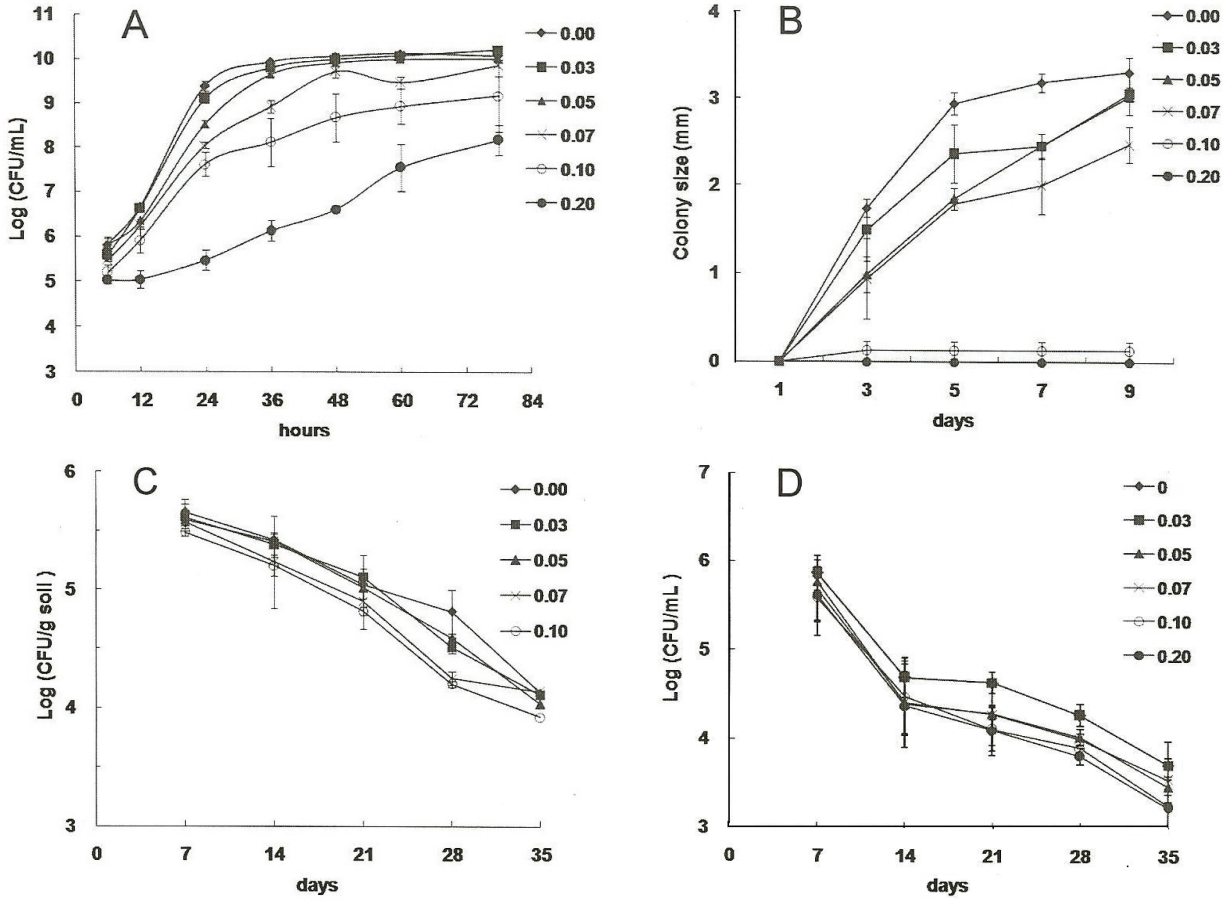


Fig. 1. Effects of salinity on the multiplication and survival of *R. solanacearum* Pss4. A: Multiplication in 523 liquid medium. B: Multiplication in 523 solid medium. C: Survival in sand. D: Survival in water. Growth and survival of *R. solanacearum* were assessed in 0 M NaCl (◆), 0.03 M NaCl (■), 0.05 M NaCl (▲), 0.07 M NaCl (×), 0.10 M NaCl (○), and 0.20 M NaCl (●).

titers were determined weekly by serial dilutions and plating on modified SM1 medium. Data collected from at least two independent trials were analyzed.

RESULTS

Effects of salinity on the multiplication and survival of *R. solanacearum* Pss4

To evaluate effects of salinity on bacterial population dynamics, we assessed bacterial multiplication and survival at various concentrations of NaCl under different experimental systems (Fig. 1). In liquid rich medium, salinity had a detrimental effect on bacterial multiplication at a dose-dependent manner (Fig. 1A). Bacterial multiplication in the presence of 0.03 and 0.05 M NaCl was not significantly different from that in control medium. Sodium chloride at a concentration of 0.07 M or higher started to have a noteworthy deleterious effect on bacterial growth, particularly at early growth stage. However, although bacterial growth rate was significantly affected by salinity, the multiplication of the bacterium was not completely inhibited. When the test was carried out in solid rich medium, salinity caused a similar but more severe negative effect on bacterial multiplication as compared to that occurred in liquid rich medium (Fig. 1B). A noteworthy deleterious effect was observed starting at a concentration of 0.03 M, and a concentration at 0.10 M or higher even completely suppressed bacterial multiplication.

To further evaluate effects of salinity on bacterial population dynamics under conditions reflecting natural environments, we assessed bacterial survival at various concentrations of NaCl in sand and in water. The results showed that, although dose-dependent deleterious effects remained, all the test salinity doses affected bacterial survival, if there was any, only very slightly in both systems (Figs. 1C and 1D).

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Effects of excess copper on the multiplication and survival of *R. solanacearum* Pss4

Effects of excess copper on bacterial population dynamics were investigated by analyzing bacterial multiplication and survival in the presence of various concentrations of $CuSO_4$ under different experimental systems (Fig. 2). In liquid rich medium, excess $CuSO_4$ had a negative effect on bacterial multiplication at a dose-dependent manner (Fig. 2A). Bacterial multiplication in the presence of up to 2.0 M $CuSO_4$ was not significant different from that in control medium, whilst bacterial growth in 4.0 M $CuSO_4$ was severely reduced but not completely inhibited. When the test was carried out in solid rich medium, excess $CuSO_4$ caused a similar but more severe negative effect on bacterial multiplication as compared to that occurred in liquid rich medium (Fig. 2B). A substantial deleterious effect was observed starting at a concentration of 0.25 mM, and a concentration at 4.0 M completely suppressed bacterial multiplication. When effects of excess copper on bacterial survival in sand were examined, the results showed that all of the test $CuSO_4$ concentrations had a negative but not very severe effect (Fig. 2C).

Effects of extreme pHs on the multiplication of *R. solanacearum* Pss4

To study effects of extreme initial pHs on bacterial population dynamics, bacterial multiplication at various pHs was assessed (Fig. 3). In liquid rich medium at various initial pHs, the bacterium grew rapidly with a order as: pH 6.0/7.0 > pH 8.0 > pH 5.0 (Fig. 3A). A growth condition at pH 9.0 severely reduced bacterial multiplication. The influence of extreme pHs was more apparent at the early stage of bacterial growth. When the test was carried out in solid rich medium, extreme pHs caused a similar but more severe negative effect on bacterial multiplication as compared to that occurred in liquid rich medium (Fig. 3B). A noteworthy deleterious effect on bacterial multiplication was observed at pH 5.0 in solid medium, and the bacterial growth was even completely suppressed at pH 9.0.

Effects of drought and incident light on the survival of *R. solanacearum* Pss4

Effects of drought and incident light on bacterial population dynamics were evaluated by determining bacterial survival in sand and in water, respectively. As shown in Fig. 4A, the bacterial titer declined considerably slowly with adequate humidity during the period of time in our experiments (five weeks), but reduced rapidly under drought condition, reaching to the bottom level within 3 weeks. Furthermore, during the duration of experiments, the bacterial titer in water remained quite constant in dark as well as under incident light (Fig. 4B).

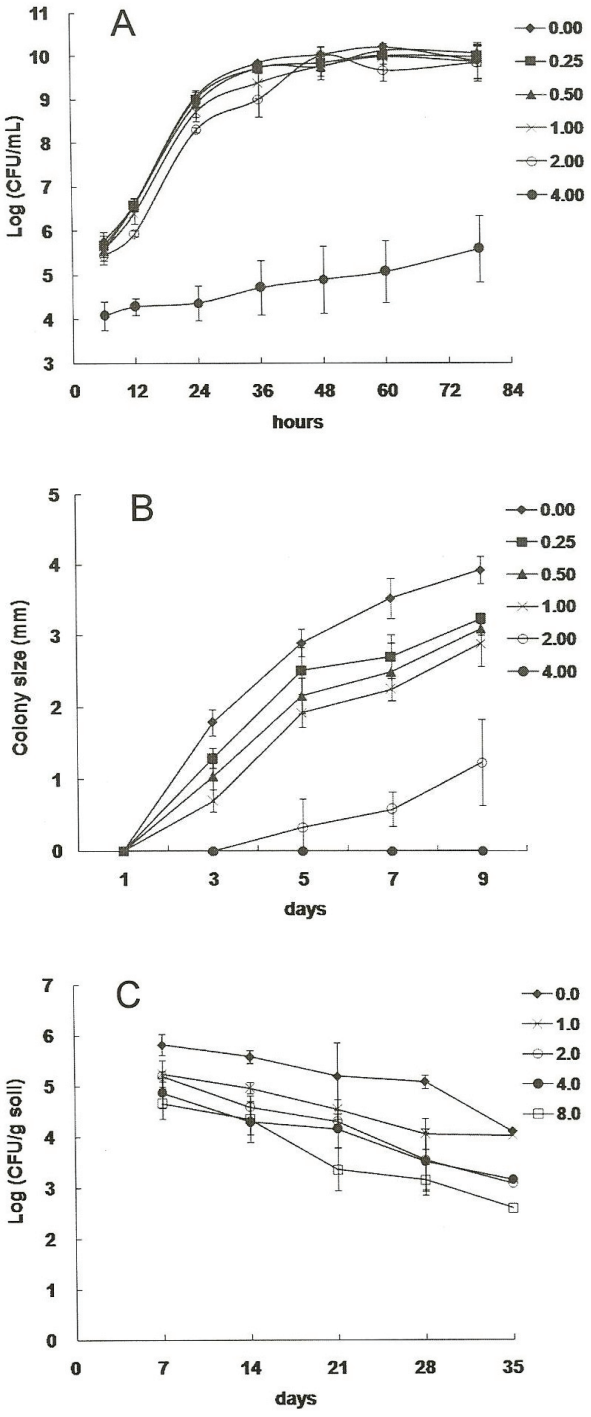


Fig. 2. Effects of excess copper on the multiplication and survival of *R. solanacearum* Pss4. A: Multiplication in 523 liquid medium. B: Multiplication in 523 solid medium. C: Survival in sand. Multiplication and survival of *R. solanacearum* were assessed in 0 mM $CuSO_4$ (◆), 0.25 mM $CuSO_4$ (■), 0.5 mM $CuSO_4$ (▲), 1,0 mM $CuSO_4$ (×), 2.0 mM $CuSO_4$ (○), 4.0 mM $CuSO_4$ (●) and 8.0 mM $CuSO_4$.

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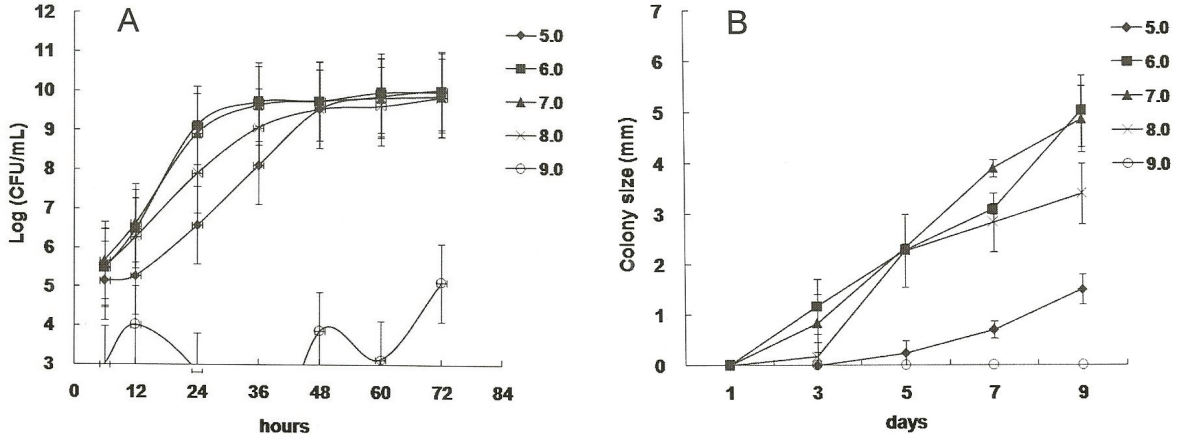


Fig. 3. Effects of extreme initial pHs on the multiplication of *R. solanacearum* Pss4. A: 523 liquid medium. B: 523 solid medium. Multiplication of *R. solanacearum* were assessed at pH 5.0 (◆), pH 6.0 (■), pH 7.0 (▲), pH 8.0 (×), and pH 9.0 (○).

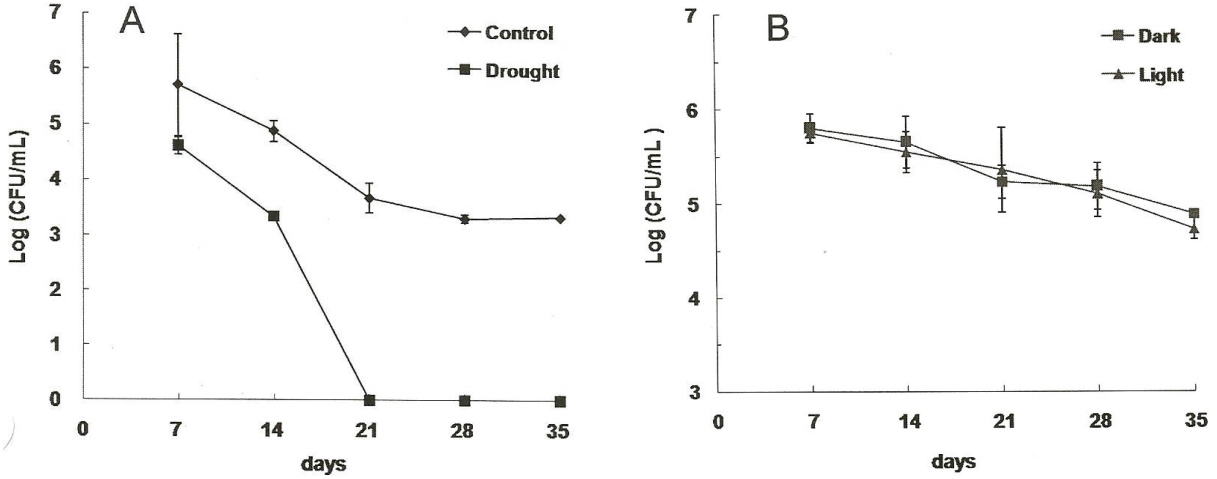


Fig. 4. Effects of drought and incident light on the survival of *R. solanacearum* Pss4. A: Drought tests in sand. B: Incident light tests in water. Conditions of drought and light treatment were described in methods.

DISCUSSION

Microbes encounter various environmental factors which frequently cause deleterious effects on their population dynamics. Previous reports mostly carried out tests on regional *R. solanacearum* strains in natural microcosms of local areas (Michel and Mew, 1998; van Elsas et al., 2001; Caruso et al., 2005; Àlvarez et al., 2007; Messiha et al., 2007). Due to the complex properties among different regions and among *R. solanacearum* strains, the results certainly cannot be applied to Taiwan directly. In this study, we initially characterized effects of important environmental factors on the propagation and survival of a local *R. solanacearum* strain, which can be representative of most *R. solanacearum* existing in Taiwan fields (Jaunet and Wang, 1999). To clearly assess effects of each of the environmental factors, this study initially evaluated individual test factors in controlled experimental conditions. In addition, to better evaluate the effects of the test factors on bacterial multiplication and survival, four assay systems were used, including liquid rich medium, solid rich medium, autoclaved sand and autoclaved water. In general, our results indicated that the growth of *R. solanacearum* in solid media was suffered from negative effects caused by the test environmental stress factors at higher levels, as compared that in liquid media (Figs. 1A, 1B, 2A, 2B, 3A, 3B). These results suggested that bacterial multiplication in the course of colony formation from single bacterial cells was more vulnerable and thus required critical conditions to complete the course.

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Additionally, except for drought stress in sand (Fig. 4A), under the experimental conditions used in this study, *R. solanacearum* population in autoclaved sand and water declined very slowly with adequate moisture, and was not or was slightly affected by the test factors at the test doses (Figs. 1C, 1D, 2C, 4B). These data together with the findings of viable-but-not cultural state of *R. solanacearum* in natural biosystems (Grey and Steck, 2001; van Overbeek et al., 2004) clearly provided good explanation for the success of this destructive bacterium in nature.

Salts are essential components for the survival and functioning of all organisms. However, optimal salt concentrations can vary among different organisms. The NaCl concentration in seawater is 3~5% (or 0.5~0.85 M). A previous report carried out on a biovar 2 strain in Spanish irrigation water showed that levels of seawater salts realistic for drainage water in coastal areas were detrimental to bacterial survival (van Elsas et al., 2001). However, effects of excess NaCl on the multiplication of *R. solanacearum* are not known. Our results showed that the multiplication of *R. solanacearum* Pss4 in rich media was significantly reduced by 0.07 M NaCl or at a higher concentration (Figs. 1A and 1B), indicating that this bacterium would be sensitive to seawater during its active propagation. However, NaCl at all of the test concentrations (0.0~0.2 M) did not cause noteworthy detrimental effects on the survival of Pss4 (Figs. 1C and 1D). Since all of the NaCl concentrations tested in our study were lower than that in real seawater, and because different *R. solanacearum* strains were studied, more studies would be necessary to further determine effects of high NaCl levels on the bacterial population dynamics in natural biosystems.

Copper is an essential trace element for life, but can become deleterious at high concentrations. Despite the toxic effect, resistance mechanisms and the genes involved against copper concentrations have been found on some bacteria (Mergeay et al., 2003; Nies, 2003; Rensing and Grass, 2003). Homologues of genes involved in copper resistance are also present in *R. solanacearum* genome (Salanoubat et al., 2002; Genin and Boucher, 2004; Gabriel et al., 2006). In a previous report, an USA strain of *R. solanacearum* was shown to enter into the viable-but-nonculturable state in response to various levels of cupric sulfate under starvation, both in a saline solution and in autoclaved soil (Grey and Steck, 2001). However, effects of excess copper on multiplication of this bacterium are not known. Consistent with the previous report, our analysis carried out in sand containing various concentrations of $CuSO_4$ showed a significant detrimental effect on culturable bacterial populations (Fig. 2C). Furthermore, our study revealed that active multiplication of this bacterium in rich media was very sensitive to the presence of $CuSO_4$, with a detrimental effect on the number of multipliable cells starting at a concentration 4 mM (Figs. 2A and 2B), highly likely because the bacterium enters into a copper-induced viable-but-nonculturable state. Bordeaus mixture, a pesticide commonly used in fields, contains 125 mM CuSO4, a level which can only cause *R. solanacearum* to enter into a viable-but-nonculturable state (Grey and Steck, 2001). In addition, the allowed copper level in industrial released pollutants in Taiwan is 3.0 mg/L (or 0.01875 mM). As copper-induced viable-but-nonculturable state of *R. solanacearum* has been shown to be involved in long-term survival of the bacterium and in plant infection (Grey and Steck, 2001), the use of copper-based pesticides for disease control of bacterial wilt not only is an impractical strategy, but also will cause the irreversible harm to our ecosystems.

Microbes confer differential tolerance to extreme pHs. Some have even evolved to live optimally in extreme pHs. A previous study on the effect of a soil amendment on the survival of *R. solanacearum* in four Philippine soils suggested that a decrease of bacterial population occurred in a soil with a basic pH (Michel and Mew, 1998). Additionally, this study demonstrated that ammonium reduced growth of *R. solanacearum* only at pH 9.0 and nitrite was suppressive only at pH 5.0. However, effects of extreme pHs on multiplication of this bacterium have not been studied. In this study, our results further showed that *R. solanacearum* Pss4 propagates optimally at pH 6.0 and 7.0 under nutrient-sufficient conditions, and to a less degree at pH 8.0 (Fig. 3). Nevertheless, the bacterial multiplication was severely reduced at pH 9.0 in both liquid and solid rich media and at pH 5.0 in a solid medium. As the pH of xylem sap from tomato plants is approximately 5.0 (Wilkinson et al., 1998), how *R. solanacearum* achieves optimal propagation in plant tissues remains unclear.

In nature, *R. solanacearum* can survive in soil over a long time and may associate with infection of new plants (Grey and Steck, 2001). Our data showed that *R. solanacearum* Pss4 population in sand sustained quite well with adequate moisture under the experimental conditions used in this study, but declined very rapidly upon drying (Fig. 4A). Consistently, a previous study reported that a biovar 2 strain, which was considerably persistent in canal sediment saturated with drainage water, died out quickly when subjected to drying (van Elsas et al., 2001). These results together reveal that the survival of *R. solanacearum* in is considerably sensitive to drought.

R. solanacearum biovar 2 strains can be relatively frequently detected in water courses in Europe (van Elsas et al., 2001; Caruso et al., 2005; Àlvarez et al.,

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2007). It is thus reasonable to assume this bacterium is also present in water courses in Taiwan. Previously, it has been reported that a light-dark regime can negatively affect the survival of a *R. solanacearum* biovar 2 strain in natural drainage water (van Elsas et al., 2001). However, we did not find a similar effect of an incident light on the survival of Pss4 in autoclaved water during the period of experiments under the defined conditions (Fig. 4B). Two reasons may non-exclusively account for the differences between our and other reports. Firstly, many abiotic and biotic factors, individually or together with various interactions, can affect bacterial survival in natural systems at various levels (Michel and Mew, 1998; van Elsas et al., 2001; Caruso et al., 2005; Àlvarez et al., 2007; Messiha et al., 2007). Thus, the use of different bioassay systems (pure autoclaved water in our study *vs.* natural drainage water in the other studies) may result in the inconsistent observations. Secondly, due to the complex nature of *R. solanacearum* strains, Pss4 may truly respond to light differently from biovar 2 strains. Further studies are required to make clear of these questions.

In conclusion, our study has set up to initially evaluate effects of important environmental factors on the propagation and survival of a representative local *R. solanacearum* strain under laboratory assay systems. Although many abiotic and biotic factors in natural biosystems can together affect bacterial survival at various levels, the assay systems established in this work and the information gathered from this study are expected to pave to the way for further studies on the field samples under natural conditions. Furthermore, a very interesting and important question worthy of further study is: in addition to copper, whether can other extreme environmental factors induce the development of a viable-but-nonculturable state of *R. solanacearum* in laboratory conditions and in natural biosystems? Additionally, much more information regarding mechanisms and genes involved in common and differential tolerance to various extreme environmental factors in different *R. solanacearum* strains remain to be explored. Putting all together, the long-term goal of studies on effects of environmental factors on the multiplication and survival of *R. solanacearum* is to hopefully benefit advancement of disease epidemiology and development of feasible strategies for disease control.

ACKNOWLEDGEMENTS

We thank Kuan-Ying Hwang and Ming-Long Cheng for technical assistance at the initial stage of this study. This work was supported by a research grant (NSC96-2313-B-002-055-MY2) from the National Science Council, Republic of China (to C.-P. Cheng).

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環境因子對於臺灣番茄青枯病菌株增殖與存活之影響

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(收稿日期：2008 年 8 月 25 日；接受日期：2008 年 11 月 9 日)

摘要：在自然環境中，微生物常遭遇對其族群動態有害的因子。植物青枯病菌 (*Ralstonia solanacearum*) 是一種可造成眾多重要作物致死萎凋病的複雜病原細菌，為了尋求有效的病害防治策略，全面性地了解自然環境因子對於青枯病菌族群動態的影響自然是十分重要且必需的，但目前人們對於這方面所知仍十分有限，且所得訊息常是各地而異。在本研究中，我們針對一株具地區代表性的臺灣番茄青枯病菌株，分析高鹽、過量銅、極端 pH 值、乾旱及光照等因子對此菌之增殖與存活的影響。結果顯示：(1)在優養條件下，高鹽對此菌的增殖有不良影響，但對於在沙土或水中的病菌存活則並無顯著的影響；(2)過量銅對優養條件下的病菌增殖有不良影響，而對在沙土中病菌存活的影響則較小；(3)在 pH 值為 9.0 或 5.0 的優養條件下，病菌的繁殖大大降低；(4) 乾旱對在沙土中病菌的存活影響極大，而光照對病菌在水中存活的影響則並不顯著。此外，我們也進一步將這些結果與在全球其他地區針對當地青枯病菌菌株所得之結果進行比較分析。本研究所得之資訊與建立之分析系統，將有助於未來全面性分析臺灣田間樣本中青枯病菌之族群動態及環境因子對其增殖與存活的影響，並期望對於青枯病流行病學及防治策略有所助益。

關鍵詞：青枯病菌、增殖、存活、環境因子。

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