Journal of Experimental Sciences 2012, 3(9): 05-09
ISSN: 2218-1768
Available Online: http://jexpsciences.com/

journal of experimental sciences

Aggressiveness of *Ralstonia solanacearum* isolates on Tomato

K. N. Chandrashekara[1*], M. K. Prasanna Kumar[2] and S. Saroja[3]

[1]Division of Biotechnology, Indian Institute of Horticultural Research, Hessaraghatta Lake post, Bangalore-560 089, India
[2]Department of Plant Pathology, University of Agricultural Science, GKVK, Bangalore-560 064, India
[3]Division of Entomology and Nematology, Indian Institute of Horticultural Research, Hessaraghatta Lake post, Bangalore-560 089, India

Abstract

Bacterial wilt caused by *Ralstonia solanacearum* is the world's most economically important destructive disease of crop plants. Fifty seven isolates of *R. solanacearum* causing wilt on different host plants were collected from different agro climatic zones of India of which 54 isolates were confirmed as race-1, biovar-3 and 3 isolates as race-1, biovar-3B based on morphological, physiological, biochemical and pathogenicity studies. All the isolates were authenticated as *Ralstonia solanacearum* by using two sets of primers (OLI1 & Y2 and Y1 & Y2). Serological identity of the isolates was done by using diagnostic kit obtained from International Potato Research Center, Lima, Peru and single chain variable fragment antibody specific to *Ralstonia solanacearum.* Fifty seven isolates of *R. solanacearum* were inoculated on tomato (*Solanum Lycopersicum* L) cv. Avinash-II under artificial conditions at bacterial concentration of $5x10^8$ cfu/ml to test its aggressiveness; the results obtained are discussed in this paper.

Keywords: *Ralstonia solanacearum*, isolates, tomato, aggressiveness, wilt

INTRODUCTION

Ralstonia solanacearum (*Pseudomonas solanacearum*) (E.F. Smith) [1] as a species has extremely wide host range, but differ in pathogenic varieties (races) within the species may show more restricted host ranges. Several hundred species of tropical, subtropical and warm temperature plants are susceptible to one or other races of *R. solanacearum* causing heavy losses in many economically important crops [2, 3]. The species is highly heterogeneous and complex. It is a major constraint in the production of many important vegetables, fruit, and cash crops. It is a devastating soil-borne plant pathogen with a global distribution and an unusually wide host range. It causes wilt in more than 450 host species in 54 botanical families [4] which may give the pathogen an evolutionary advantage and that number of new species continues to increase [2].

MATERIALS AND METHODS

Isolation of *R. solanacearum* strains from field

Wilt affected host plants *viz*., tomato, brinjal, potato, bird of paradise, ginger, capsicum, chilli, davana and coleus showing typical symptoms of wilt were collected from different agro climatic zones of Karnataka and other parts of India (Table 1). Bacterium was isolated on SMSA media [5, 6]. The isolates were subjected to identification and confirmation based on the morphological, physiological, cultural, biochemical and pathogencity studies [7]. The isolates were differentiated into biovars [8, 2].

Received: June 05, 2012; Revised: Aug 10, 2012; Accepted: Nov 26, 2012.

*Corresponding Author

K. N. Chandrashekara
Division of Biotechnology, Indian Institute of Horticultural Research,
Hessaraghatta Lake post, Bangalore-560 089, India

Email: knchandu1@gmail.com

Sugar utilization test

The isolates were differentiated into biovars [2] using carbohydrate fermentation. All tests *viz.,* starch hydrolysis, nitrate reduction, oxidase, indole production, esculin hydrolysis, arginine dihydrolase, curdling of skimmed milk were carried out as per the methods described in the Manual of Microbial Methods [9] and Laboratory guide for identification of Plant Pathogenic Bacteria [8].

Genomic DNA extraction, PCR Amplification and Serological detection

Genomic DNA extraction was done according to protocol described by Chandrashekara et al., 2011 [11], PCR amplified using two sets of primers corresponding to 16S rDNA (OLI1 and Y2) and 16S rRNA (Y1 and Y2) as described by Seal et al., 1993 [12] using universal primers. Further, serological confirmation was carried out by using a kit (CIP's post-enrichment DAS-ELISA kit, International Potato Center, Lima, Peru [13]. Monoclonal SCFV antibody specific to *R. solanacearum* race-1, biovar-3 was used for ELISA for confirmation of bacteria as described by Chandrashekara et al., 2011 [11].

Aggressiveness of isolates on tomato plant

Fifty seven isolates of *R. solanacearum* were multiplied in casein peptone glucose (Casamino acid 1g L^{-1}, Peptone 10g L^{-1}, Glucose 10g L^{-1} pH 7.2) to inoculate on tomato under artificial conditions. The bacterial suspension was adjusted to concentration of $5x10^8$ cfu/ml using spectrophotometer readings (Spectronic 20 D, M and R, USA) at a wave length of equivalent to A600nm =0.8 to 1.0. The susceptible tomato (*Solanum Lycopersicum* L) cv. Avinash-II, seedlings were grown in greenhouse under artificial conditions. Twenty days old seedlings of tomato were pulled out gently, washed free of soil and few tertiary roots were clipped with sterilized scissors and dipped in bacterial culture for 10 minutes. The inoculated

seedlings were then transplanted to plastic bags containing sterilized soil. Plants similarly inoculated with sterile water served as control. Observations were made on number of days taken for symptom expression.

Once the wilt symptoms appeared, the plants were allowed for two days for complete wilting. Then the plant was pulled out from the pot and 5 cms stem above the soil level was cut with sterile scalpel. One hundred mg of infected tissue was collected and immersed in 1ml of sterile distilled water for 10 minutes. Then it was used for plating on SMSA medium and observations were recorded for CFU of bacteria.

RESULTS AND DISCUSSION

Plants showing typical symptoms of bacterial wilt [7] were collected from field. Isolation of the bacterium was made; tentative diagnosis of the diseased plants was made by water streaming test [14] bacterial ooze was seen from the bits of infected plants. The bacterial ooze was subjected to morphological studies revealed that the isolates were Gram negative rod shaped, non-capsulated and non-spore forming. The bacteria grown on SMSA medium [5, 6] were found similar in characteristics with *R. solanacearum*. The colonies were highly fluidal, white colored with light pink center, bluish margin with round to irregular shape.

Biovars of *R. solanacearum* are primarily differentiated as per [2]. The isolate produced typical wilt symptoms on its respective host within 3 to 4 weeks time after inoculation by root injury technique [15]. Thus, based on morphology, host range, ability to cause wilt to solanaceous & non solanaceous crops and pathogenicity, the isolate was confirmed as race-1, biovar -3/3B. The isolates were negative to indole and gelatin lignifications, positive for oxidase, levan production, nitrate reduction and didn't produce fluorescence on Kings B media. Our findings are in accordance with earlier reports [2, 16].

Partial sequences of 16S rDNA and 16S rRNA genes are excellent targets for identification of bacteria at the species level as they are species-specific and available in multiple copies in microbial genome. The corresponding specific rDNA and rRNA sequences have been used as targets for PCR amplification [17]. The PCR amplification resulted in 300bp and 292bp product from davana isolate. This was confirmation of the earlier results by Seal [12] for *R. solanacearum*.

Detection of *R. solanacearum* by DAS-ELISA kit originally developed to detect latent infection in potato seed tubers was adopted to authenticate the isolates showed positive reaction with bright purple coloration on nitrocellulose membrane that was compared with positive control strips provided in the kit [13]. Further the scFv monoclonal antibody (anti-R solanacearum-ALP conjugate) detected *R. solanacearum*. This was for the first time a scFv antibody has been developed to detect *R. solanacearum* race-1, biovar-3 [11]. We undertook this study to better understand the interactions between tomato plant and the different isolates of *R. solanacearum* R1bv3/3B.

These host pathogen interactions are of particular concern to breeders and tomato producers working to develop resistant varieties to a particular geographical condition. *R. solanacearum* is a relevant and widespread phytopathogenic bacterium that causes a wilt disease with deadly effects on many economically important crops. This soil and water borne bacterium enters the plant roots, multiplies through the xylem, collapses the host and returns to the environment. Once in the plant tissues, high densities of the pathogen increase expression of virulence genes and production of exopolysaccharide, the main pathogenicity determinant. These genes are controlled by a density-dependent regulatory network taking part in a quorum sensing system [18]. After destroying the plant, the bacterium can spread by infected plants by irrigating with sub-irrigation or ebb-and-flow systems. The bacterium can also enter plants by way of stem injuries from insects, handling or tools [19]. Jaunet and Wang [20] had hypothesized that the location specificity of resistance observed in the worldwide evaluation [21] could be related to the large variation in aggressiveness. The large variability observed in those populations of *R. solanacearum* can also be related to its soil borne nature. The pathogen has to survive with the variable surroundings in the soil during its saprophytic survival [2]. Thus, soil properties may play an important role in genetic differentiation, which has been demonstrated for other soil borne bacterial species [22]. Furthermore, genetic recombination and horizontal gene exchange may play an important role in the genetic differentiation of soil borne bacteria [23]. Although *R. solanacearum* is competent for natural genetic transformation [24], the importance of genetic recombination on shaping population structure remains to be determined. Host plant resistance has not been very effective for control of bacterial wilt on tomato, since resistance often varies among locations because of strain differences [25, 26]. In quest to understand the variation among different isolates of *R. solanacearum* on tomato, the study was conducted. The aggressiveness of the *R. solanacearum* isolates was studied by artificial inoculation on tomato (*Solanum Lycopersicum* L.) cv. Avinash-II indicated, among 57 isolates of *R. solanacearum* tested, KERG-1 expressed wilt symptoms in a short duration (15 days) with bacterial population of $3.81X10^5$ and the isolate KERG-2 originating from same location produced wilt symptoms after 16 days with the inplanta population of $5.73X10^5$ CFU/ml followed by HRP-10 from Kandli, Hassan which expressed wilt symptoms in 19 days with the population of $6.12X10^7$ CFU/ml. While, rest of the isolates took longer to express the symptoms (20-27 days) with bacterial population ranging from $7.80X10^6$ to $7.32X10^7$CFU/ml. (Table 1 and Figure 1). It is interesting to note that isolates from Kerala expressed the wilting symptoms early and bacterial population was also low and hence more aggressive. Pathogen virulence and aggressiveness to the host is significant than the population density to cause wilt. In support to our results Kumar and Sarma [10] have reported that Kerala isolates of *R. solanacearum* are highly pathogenic to ginger plants when artificially inoculated. In our observation, we presume isolates which produce early wilt symptoms will kill the host plant and have more dispersal ability to infect nearby plants after attaining ample population causing huge loss to farmers due to its rapid spread.

CONCLUSION

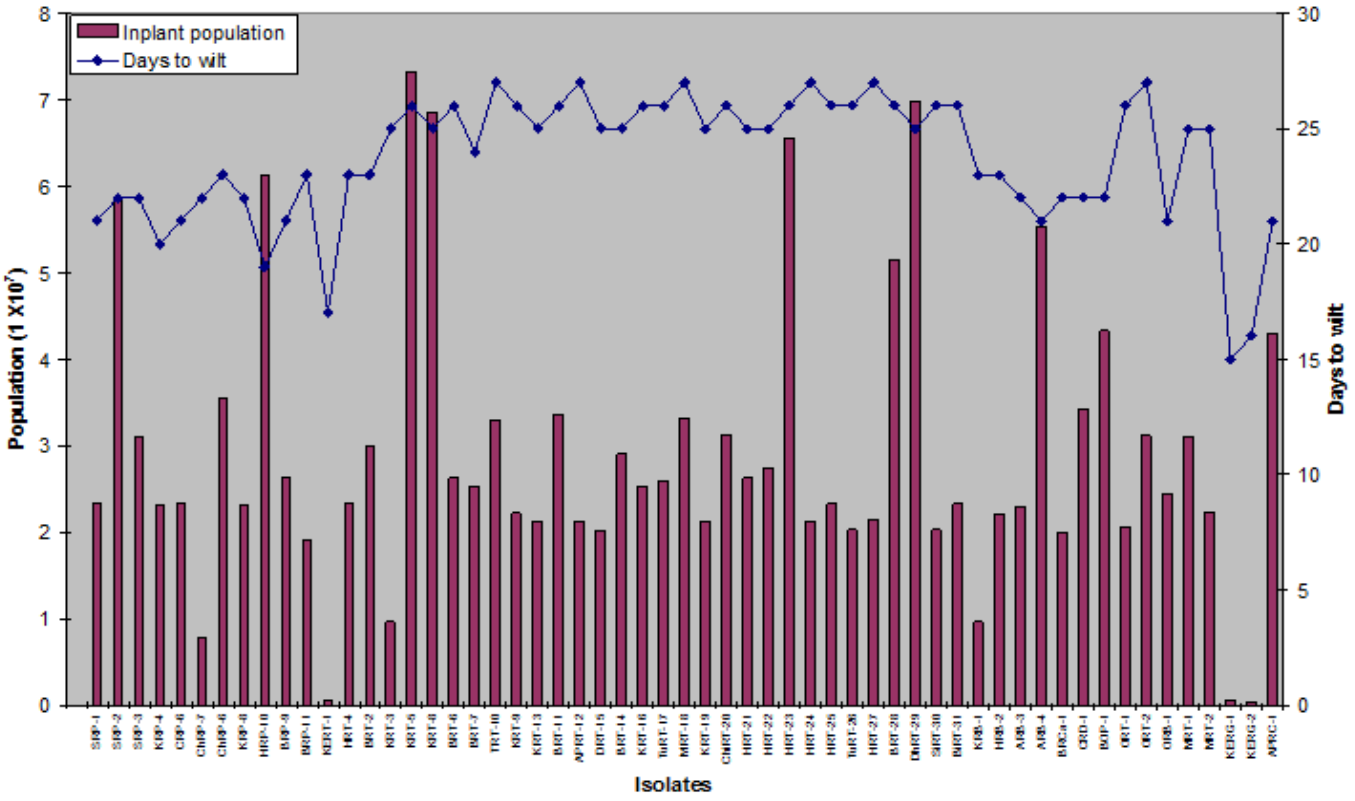
Aggressiveness trait of the pathogen needs to be studied while screening the varieties for their resistance to bacterial wilt. Till date not much work has been carried out in this aspect, hence further work with respect to cause behind variations in the aggressiveness of the bacterium will be of great use in management of *Ralstonia solanacearum*.

Table 1: Aggressiveness of *Ralstonia solanacearum* isolates on tomato

Sl. No.	Isolate code	Host	Place	Time taken to wilt (DAP)	Inplant population
1	SRP-1	Potato	*Simla (GSC-26)	21	$2.33X10^7$
2	SRP-2	Potato	*Simla (134)	22	$5.83X10^7$
3	SRP-3	Potato	*Simla (JGS 3/118)	22	$3.11X10^7$
4	KRP-4	Potato	Sulebele, Kolar	20	$2.32X10^7$
5	CRP-6	Potato	Kolar	21	$2.33X10^7$
6	ChRP-7	Potato	Chikkabalapur	22	$7.80X10^6$
7	ChRP-6	Potato	Chikamaglur	23	$3.55X10^7$
8	KRP-8	Potato	Bagepalli, Kolar	22	$2.32X10^7$
9	HRP-10	Potato	Kandli, Hassan	19	$6.12X10^7$
10	BRP-9	Potato	Chikamaglur	21	$2.63X10^7$
11	BRP-11	Potato	Bangalore	23	$1.92X10^7$
12	KERT-1	Tomato	Kerala	17	$4.93X10^5$
13	HRT-4	Tomato	Hassan	23	$2.33X10^7$
14	BRT-2	Tomato	Bangalore	23	$2.99X10^7$
15	KRT-3	Tomato	GKVK, Bangalore	25	$9.63X10^6$
16	KRT-5	Tomato	Chintamani, Kolar	26	$7.32X10^7$
17	KRT-8	Tomato	Sreenivaspura, Kolar	25	$6.86X10^7$
18	BRT-6	Tomato	Hebbal, Bangalore	26	$2.63X10^7$
19	BRT-7	Tomato	Bangalore	24	$2.53X10^7$
20	TRT-10	Tomato	Hossur, Tamil Nadu	27	$3.30X10^7$
21	KRT-9	Tomato	Gouribidnur, Kolar	26	$2.22X10^7$
22	KRT-13	Tomato	Chintamani, Kolar	25	$2.12X10^7$
23	BRT-11	Tomato	Hoskote, Bangalore	26	$3.36X10^7$
24	APRT-12	Tomato	Anantapur (AP)	27	$2.12X10^7$
25	DRT-15	Tomato	Davangere	25	$2.02X10^7$
26	BRT-14	Tomato	Anekal, Bangalore	25	$2.90X10^7$
27	KRT-16	Tomato	Kaivara, Kolar	26	$2.53X10^7$
28	TuRT-17	Tomato	Tumkur	26	$2.60X10^7$
29	MRT-18	Tomato	Mysore	27	$3.32X10^7$
30	KRT-19	Tomato	Sreenivaspura	25	$2.12X10^7$
31	ChiRT-20	Tomato	Chitradurga	26	$3.13X10^7$
32	HRT-21	Tomato	Madenur, Hassan	25	$2.63X10^7$
33	HRT-22	Tomato	Bhuvaneshwar, Orissa	25	$2.75X10^7$
34	HRT-23	Tomato	Arkalgud, Hassan	26	$6.56X10^7$
35	HRT-24	Tomato	Kandli, Hassan	27	$2.12X10^7$
36	HRT-25	Tomato	Hassan	26	$2.33X10^7$
37	TuRT-26	Tomato	Tumkur	26	$2.03X10^7$
38	HRT-27	Tomato	Hassan	27	$2.14X10^7$
39	BRT-28	Tomato	Kanakpura	26	$5.16X10^7$
40	DhRT-29	Tomato	Dharwad	25	$6.99X10^7$
41	SiRT-30	Tomato	Sirsi	26	$2.03X10^7$
42	BiRT-31	Tomato	Bijapur	26	$2.33X10^7$
43	KRB-1	Brinjal	Kolar	23	$9.66X10^6$
44	HRB-2	Brinjal	Kandli, Hassan	23	$2.21X10^7$
45	ARB-3	Brinjal	Arabhavi	22	$2.30X10^7$
46	ARB-4	Brinjal	Arabhavi	21	$5.53X10^7$
47	BRCa-1	Capsicum	GKVK, Bangalore	22	$2.00X10^7$
48	CRD-1	Davana	Chikkabalapur	22	$3.42X10^7$
49	BOP-1	Bird of Paradise	GKVK, Bangalore	22	$4.33X10^7$
50	ORT-1	Tomato	Bhuvaneshwar, Orissa	26	$2.06X10^7$
51	ORT-2	Tomato	Bhuvaneshwar, Orissa	27	$3.12X10^7$
52	ORB-1	Brinjal	Bhuvaneshwar, Orissa	21	$2.45X10^7$
53	MRT-1	Tomato	Nasik, Maharastra	25	$3.11X10^7$
54	MRT-2	Tomato	Nasik, Maharastra	25	$2.23X10^7$
55	KERG-1	Ginger	Trissur. Kerala	15	$5.73X10^5$
56	KERG-2	Ginger	Trissur. Kerala	16	$3.81X10^5$
57	APRC-1	Coleus	Tirupati (AP)	21	$4.30X10^7$

*Indicates reference strains used in the study provided by Central Potato Research Institute, Shimla, Himachal Pradesh, India.

Fig. 1: Aggressiveness of Ralstonia solanacearum isolates



REFERENCES

[1] E. Yabuuchi, Y. Kosako, I. Yano, H. Hotta and Y. Nishiuchi. 1995. Transfer of two Burkholderia and an Alcaligenes species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Douderoff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. & *Ralstonia eutropha* (Davis 1969) comb. nov., Microbiology and Immunology. 39: 897-904.

[2] A. C. Hayward. 1991. Biology and Epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annual Review of Phytopathology. 29: 65-87.

[3] K. N. Chandrashekara and M. K. Prasannakumar. 2010. New host plants for *Ralstonia solanacearum* from India. Plant Pathology. Doi:10.1111/j.1365-3059.2010.02358.x.

[4] Allen, C., Prior, P and Hayward, A. C. 2005. Bacterial Wilt Disease and the *Ralstonia solanacearum* Species complex. APS Press: St Paul, MN, USA.

[5] J. G. Elphinstone, H. M. Stanford and D. E. Stead. 1998. Detection of *Ralstonia solanacearum* in potato tubers, *Solanum dulcamara*, and associated irrigation water. In: Bacterial Wilt Disease: Molecular and Ecological Aspects (Ed. P. Prior, C. Allen, J. Elphinstone. Springer Verlag, Berlin (DE).133-139.

[6] M. Wenneker, M. S. W. Verdel, A. R. Van Beuningen, J. H. J. Derks and J. D. Janse. 1999. *Ralstonia (Pseudomonas) solanacearum* race 3 (biovar 2) in surface water and natural weed hosts: first report on stinging nettle (Urticadioica). European Journal of Plant Pathology. 105:307-315.

[7] A. Kelman. 1954. The relationship of pathogenicity of *Pseudomonas solanacearum* to colony appearance in tetrazolium medium. Phytopathology. 44: 693-695.

[8] N. W. Schaad. 1988. Laboratory Guide for Identification of Plant Pathogenic Bacteria. APS Press, St. Paul, Minnesota.

[9] Anonymous. 1957. Manual of Microbial Methods (Ed). Society of American Bacteriologists, Mc Graw, New York.

[10] A. Kumar and Y. R. Sarma. 2004. Characterization of *Ralstonia solanacearum* causing bacterial wilt in ginger. Indian Phytopathology. 57 (1):12-17.

[11] K.N. Chandrashekara, M. K. Prasannakumar, M. Deepa and A. Vani. 2011. Coleus, A new host for *Ralstonia solanacearum* race 1 biovar 3 in India. Journal of Plant Pathology. 93 (1), 233-235

[12] S. E. Seal, L. A. Jackson, J. P. W. Young and M. J. Daniels. 1993. Differentiation of *P. solanacearum*, *P. syzygii, P. pickettii* and the blood disease bacterium by partial 16S RNA sequencing, construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. Journal of General Microbiology. 139: 1587-1594.

[13] S. Priou, L. Gutarra, H. Fernandez and P. Aley. 1999. Highly sensitive detection of *Ralstonia solanacearum* in latently infected potato tubers by post enrichment ELISA on nitrocellulose

membrane. EPPO-OEPP Bulletin. 29: 117-125.

[14] C. Danks and I. Barker. 2000. On-site detection of plant pathogens using lateral flow devices. Bulletin OEPP/EPPO Bulletin 30: 421-426.

[15] J. F. Wang and T. Berk. 1997. Sources of resistance to Bacterial Wilt in *Capsicum annuum*. ACIAR Bacterial Wilt Newsletter 14: 3 - 4.

[16] Mitsuo Horita and Kenichi Tsuchiya. 1999. Phenotypic Characteristics and Cluster Analysis of Japanese and Reference Strains of *Ralstonia solanacearum*. Annual Phytopathology Society of Japan 65: 604-611.

[17] C. R. Woese. 1987. Bacterial Evolution. Microbiology Review 51: 221-271.

[18] Belén Álvarez, Elena G. Biosca and María M. López. 2010. On the life of *Ralstonia solanacearum*, a destructive bacterial plant pathogen. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. A. Mendez Vilas (Ed) Pp. 267-279.

[19] Janse, J. 1996. Potato brown rot in Western Europe – history, present occurrence and some remarks on possible origin, epidemiology and control strategies. Bulletin OEPP/EPPO 26: 679- 695.

[20] Jaunet, T. X and Wang, J. F. 1999. Variation in Genotype and Aggressiveness of *Ralstonia solanacearum* Race 1 Isolated from Tomato in Taiwan. Phytopathology. 89(4): 320-327.

[21] Wang, J. F., Hanson, P and Barnes, J. A. 1998. Worldwide evaluation of an international set of resistance sources to bacterial wilt in tomato. Pages 269-275 in: Bacterial Wilt Disease. Molecular and Ecological Aspects. P. Prior, C. Allen, and J. Elphinstone, eds. Springer-Verlag, Berlin, Germany.

[22] McArthur, J. V., Kovacic, D. A and Smith, M. H. 1988. Genetic diversity in natural populations of soil bacterium across a landscape gradient. Proc. Natl. Acad. Sci. U.S.A. 85:9621-9624.

[23] Haubold, B and Rainey, P. B. 1997. Towards an understanding of the population genetics of plant-colonizing bacteria. Adv. Bot. Res. 24:335-351.

[24] Bertolla, F., Van Gijsegem, F., Nesme, X and Simonet, P. 1997. Conditions for natural transformation of *Ralstonia solanacearum*. Appl. Environ. Microbiol. 63:4965-4968.

[25] Hanson, P. M., Wang, J. F., Licardo, O., Hanudin, Mah, S. Y., Hartman, G. L., Lin, Y. C and Chen, J. T. 1996. Variable reactions of tomato lines to bacterial wilt evaluated at several locations in Southeast Asia. Hort. Science 31:143-146

[26] Wang, J. F., Hanson, P and Barnes, J. A. 1998. Worldwide evaluation of an international set of resistance sources to bacterial wilt in tomato. Pages 269-275 in: Bacterial Wilt Disease: Molecular and Ecological Aspects. P. Prior, C. Allen, and J. Elphinstone, eds. Springer Verlag, Berlin, Germany.