# Understanding the "cool-temperate" epidemiology of *Ralstonia solanacearum* Race 3 biovar 2 on geraniums and other hosts

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## Introduction

R3bv2 strains of the plant pathogen *Ralstonia solanacearum* cause potato brown rot disease, a major problem in the highland tropics and in some temperate zones. These strains are described as more cool-tolerant than other *R. solanacearum* strains, which are warm-temperate/tropical. Establishment of R3bv2 strains in North America could threaten the U.S. potato industry. Thus the R3bv2 subgroup is a quarantine pest and a listed Federal Select Agent. This pathogen can also infect geraniums, which are commonly produced in regions where R3bv2 is endemic. Accidental introductions of R3bv2 to North America in infected geranium cuttings disrupted ornamental production and inflicted large losses. Our goals are to improve methods for detection and exclusion of this bacterium and to better understand the biology of its spread, infection, and persistence.

## Recent Accomplishments

1. We tested the frequent assertion that R3bv2 survives cold better than other *R. solanacearum* strains. Surprisingly, we found that:

- Native U.S. *R. solanacearum* strains survive longer at 4°C than R3bv2 (Figure 1; Table 1).
- At cool temperatures, R3bv2 survives longer in plants (Figure 2) and is more virulent (Figure 3)
- Thus, its distinctive epidemiological traits do not derive from improved environmental persistence.

Milling, A., F. Meng, T. P. Denny, and C. Allen. 2009. Interactions with hosts at cool temperatures, not cold tolerance, explain the unique epidemiology of *Ralstonia solanacearum* Race 3 biovar 2. **Phytopathology** 99:1127-1134.

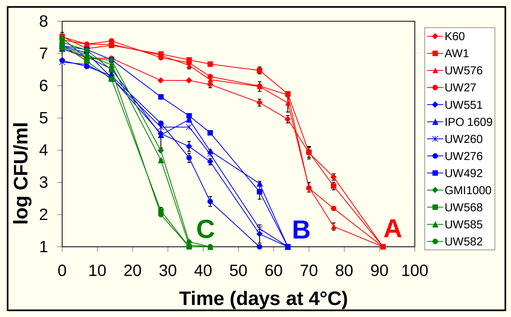
2. To identify the biological basis of the cool-temperate phenotype of R3bv2 strains, we compared gene expression between tropical and R3bv2 strains at 20°C and 28°C.

- Microarray analysis identified some R3bv2 genes differentially expressed at 20 C versus 28 C (Table 2).
- Mutant bacterial strains lacking some of these putative cold-epidemiology traits appear to be less virulent than wild-type at 20 C (Figure 4).

3. We are characterizing survival of R3bv2 in geranium, tomato, and potato tissue at sub-zero and fluctuating temperatures.

- Preliminary results indicate that even when protected by plant tissue R3bv2 cannot survive the multiple freeze-thaw cycles typical of natural environments (Figure 5).

### Fig. 1 Native US *R. solanacearum* strains survived best in water at 4 C



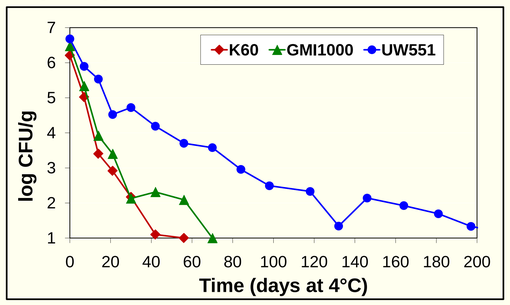

Washed bacterial cells were used to inoculate 10-ml MilliQ water microcosms to a density of 1- 2 x $10^7$ CFU/ml. Cells were starved for 2 days at RT before incubation at 4 C. Culturable cells were enumerated at regular intervals by dilution plating. Groups A, B and C were distinguished based on relative survival (see Table 1).

### Table 1 *R. solanacearum* strains studied

| Group/Strain | Race | Biovar | Phylotype[a] | Isolated from | Location |
|---|---|---|---|---|---|
| A K60 | 1 | 1 | II/7 | Tomato | N.C., USA |
| A AW1 | 1 | 1 | II/7 | Tomato | Alabama, USA |
| A UW27 | 1 | 1 | II/7 | Tobacco | Florida, USA |
| A UW576 | 1 | 1 | II/7 | Tomato | Florida, USA |
| A UW20 | 2 | 1 | II/6 | Banana | Venezuela |
| | | | | | |
| B UW551 | 3 | 2 | II/1 | Geranium | Wisconsin, USA |
| B IPO1609 | 3 | 2 | II/1 | Potato | The Netherlands |
| B UW260 | 3 | 2 | II/1 | Potato | Peru |
| B UW276 | 3 | 2 | II/1 | Potato | Mexico |
| B UW492 | 3 | 2 | II/1 | Potato | Peru |
| | | | | | |
| C UW582 | 1 | 1 | II/5 | Hydrangea | Florida, USA |
| C GMI1000 | 1 | 3 | I/12 | Tomato | French Guyana |
| C UW585 | 1 | 3 | I/13 | Pepper | Florida, USA |
| C UW568 | 1 | 3 | I/14 | Soil, tomato field | Guatemala |

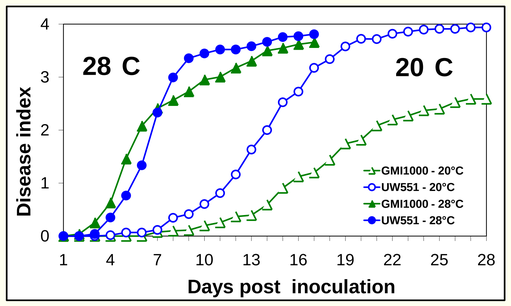
[a]Phylotype/ Sequevar. Phylotype determined by multiplex PCR and sequevar determined based on *egl* gene DNA sequence

### Fig. 2 R3bv2 strain UW551 survived longer at 4 C in potato tubers than strains native to the US (K60) and the tropics (GMI1000)



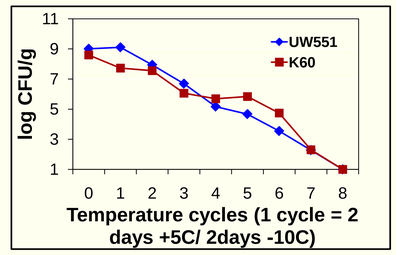

Potato minitubers (cv.'Shepody') were inoculated to an initial cell density of 1-2 x $10^6$ CFU/ tuber. Tubers were stored at 4 C, a typical storage temperature for seed potatoes. Culturable cells were enumerated at regular intervals by dilution plating. Data shown are means of two experiments.

### Fig. 3 R3bv2 strain UW551 was more virulent than tropical strain GMI1000 at 20 C



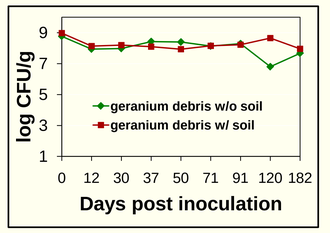

Sixteen-day-old unwounded tomato plants (cv.'Bonny Best') were inoculated by pouring bacteria onto the soil to a final concentration of about 1x$10^8$ CFU/g soil. Plants were rated daily on a 0-4 disease index scale where 0 = healthy and 4 = 100% wilted. Data shown are means of three experiments.

### Fig. 5A R3bv2 populations declined rapidly in geranium debris during freeze-thaw cycles (+5 /-10 C)

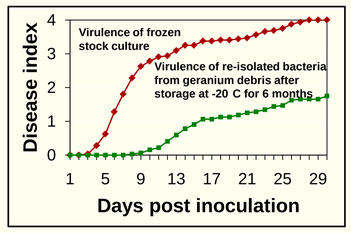



Stems of infected geraniums (containing about 1 x $10^9$ CFU/g bacteria) were chopped and subjected to varying temperatures (1 cycle = 2 days at 5°C/ 2 days -10°C). After each cycle samples were ground and plated on rich medium to determine survival of bacteria.

### Fig. 5B R3bv2 strain UW551 survived well in geranium debris at -20 C, but loses virulence




Stems of infected geraniums (containing about 1x$10^9$ CFU/gm) were chopped and stored at -20°C.




Tomato plants (cv. Bonny Best) were inoculated via the cut petiole of the first true leaf. Disease index scale: 0 = healthy, 4 = 100% wilted.

## Other Accomplishments Since 2006

- We showed that the commonly-used PCR assay for R3bv2 can give false positives; as a result APHIS instituted a more robust 2-step diagnostic protocol that combines PCR with a biovar test.

Ji, P., C. Allen, A. Sanchez-Perez, J. Yao, J G. Elphinstone, J. Jones, and T. Momol. 2007. New diversity and diagnostic challenges associated with *Ralstonia solanacearum* strains in Florida. **Plant Disease** 91:195-203.

- We showed that neither bacteriological and molecular tests can reliably detect R3bv2 in effluent water from latently infected geranium plants.

Swanson, J. K., L. Montes, L. Mejia and C. Allen. 2007. Detection of latent infections of *Ralstonia solanacearum* Race 3 biovar 2 in geraniums. **Plant Disease** 91:828-834.

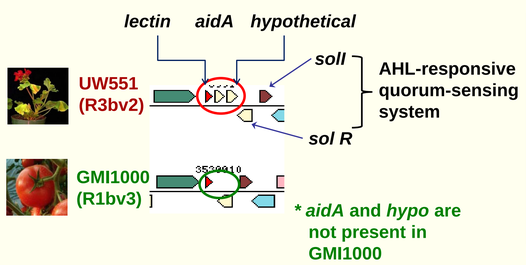
- We surveyed and characterized R3bv2 strains from the Guatemalan highlands and determined that this pathogen is not usually transmitted through tomato fruit or seed; this had an indirect effect on floriculture producers.

Sanchez-Perez, A., L. Mejia, M. Fegan, and C. Allen. 2008. Diversity and distribution of *Ralstonia solanacearum* strains in Guatemala and rare occurance of tomato fruit infection. **Plant Pathology** 57:320-331.

- Our genome sequence of *R. solanacearum* R3b2 strain UW551 is driving new research into the biology of this pathogen and especially into badly needed new diagnostic methods. We are currently field-testing a set of highly R3bv2-specific diagnostic PCR primers in combination with LAMP, a rapid, robust detection method developed by our collaborator A. Alvarez.

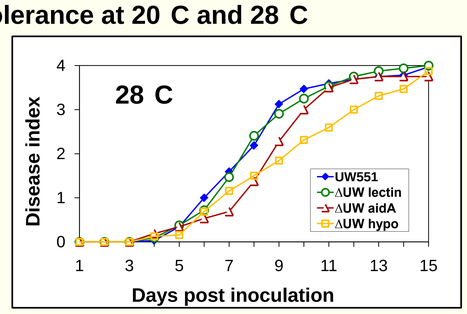
Gabriel, D. W., C. Allen, et al. 2006. Identification of Open Reading Frames unique to a Select Agent: *Ralstonia solanacearum* race 3 biovar 2. **Molecular Plant-Microbe Interactions** 19:69-79.

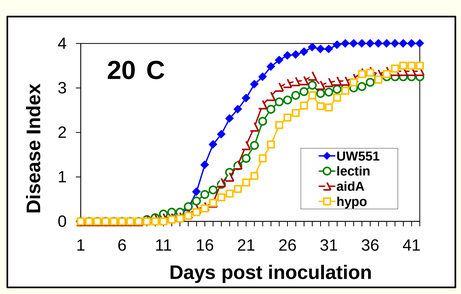
### Table 2 Gene cluster differentially expressed across strains at 20 C and 28 C




| | fold upregulation at 20 C in UW551 | |
|---|---|---|
| Genes | Microarray | qPCR |
| RRSL_02788 fucose/mannose-binding ***lectin*** | 8.6 | 39.83 |
| RRSL_02789 ***aidA*** | 12.1 | 31.60 |
| RRSL_02790 **hypothetical protein** | 3.9 | nd |
| RRSL_02791 ***solR*** | 2.4 | 2.81 |
| RRSL_02792 ***soll*** | 2.2 | nd |

### Fig. 4 Virulence of mutant strains lacking genes putatively involved in cold tolerance at 20 C and 28 C




Sixteen-day-old unwounded tomato plants (cv. Bonny Best) were inoculated by pouring bacteria onto the soil to a final concentration of about 1x$10^8$ CFU/g soil. Plants were rated daily on a disease index scale from 0 to 4 where 0 indicated healthy and 4 indicated 100% wilted.

## Technology Transfer/Impact:

- Our findings have helped shape USDA-APHIS regulations and diagnostic standards for exclusion and detection of R3bv2 in geranium cuttings (Swanson et al 2007).
- Our work quantifying the ability of R3bv2 to survive conditions in northern temperate winters has regulatory applications (Milling et al 2009).
- We have consulted regularly with CPHST scientists and floriculture industry representatives to improve offshore sanitation protocols and to resolve several false-positive reports of R3bv2 in North America (e.g. Ji et al 2007).
- We have joined efforts by the scientific community to evaluate the severity of the security threat posed by R3bv2 (Young et al 2008)
- We have worked to educate growers, regulators, and academics about the biology of this pathogen (Champoiseau et al 2009 and invited talks at various national and regional industry and academic meetings).

Champoiseau, P., J. Jones, and C. Allen. 2009. *Ralstonia solanacearum* Race 3 biovar 2 causes tropical losses and temperate anxieties. **Plant Health Progress**, published 3/13/09.

J. M. Young, C. Allen, et al. 2008. Plant pathogenic bacteria as bioterror weapons: A real threat? **Phytopathology** 98:1060-1065.

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