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Screening for *Ralstonia solanacearum* Resistance in *Solanum commersonii*

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Abstract

Worldwide, potato (*Solanum tuberosum L*) is rated as one of the most important food crops after wheat, rice and maize. Bacterial wilt, caused by *Ralstonia solanacearum* (Rs), is one of the most economically important diseases of potato. The purpose of this study was to identify diploid germplasm with resistance to bacterial wilt. The wilting degree was assessed by analyzing the wilt percentage of six *Solanum commersonii* clones which were artificially inoculated with Rs in a growth chamber. Two Rs strains (CMR15 and UW551) were used to inoculate the clones evaluate resistance. MSEE912–08, a selfed progeny from PI320266 was identified as the most resistant of the clones in this study exhibiting the lowest RAUDPC for wilting and a Wilting Degree rating of 0.4 (less than 25% wilted leaves) 20 days post inoculation when inoculated with the UW551 Rs strain, and 1.3 with the CMR15 strain. The resistance observed was characterized by reduction in wilting over time, rather than lack of disease. The most resistant clone from this study was self-pollinated to create a mapping population to identify single nucleotide polymorphic (SNP) markers associated with Rs resistance.

Resumen

A nivel mundial, la papa (*Solanum tuberosum* L.) esta catalogada como uno de los mas importantes cultivos alimenticios, después del trigo, el arroz y el maíz. La marchitez bacteriana, causada por *Ralstonia solanacearum* (Rs) es una de las enfermedades mas económicamente importantes de la papa. El propósito de este estudio fue identificar germoplasma diploide con resistencia a la marchitez bacteriana. Se evaluó el grado de marchitamiento mediante el análisis del porcentaje de marchitez de seis clones de *Solanum commersonii* que fueron inoculados artificialmente con Rs en una cámara de crecimiento. Se usaron dos sepas de Rs (CMR15 y UW551) para inocular los clones y evaluar su resistencia. El clon MSEE912–08, una progenie endogáminca de PI310266 se identificó como el mas resistente en este estudio, mostrando la RAUDPC mas baja para marchitamiento y con un Grado de Marchitez de 0.4 (menos del 25% de hojas marchitas) a los 20 días post inoculación cuando se inocularon con la variante UW551 de Rs, y 1.3 con la sepa CMR15. La resistencia observada se caracterizó por la reducción en marchitamiento en el tiempo, en vez de carencia de enfermedad. El clon mas resistente de este estudio se autopolinizó para crear un mapeo de población e identificar marcadores polimórficos de nucleótidos únicos (SNP) asociados con la resistencia a Rs.

Keywords Diploid potato · Bacterial wilt · Resistance breeding

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Introduction

Cultivated potato (*Solanum tuberosum L*) is one of the most important world food crops after wheat, rice and maize. Bacterial wilt, caused by *Ralstonia solanacearum* (Rs), is one of the most economically important diseases affecting potato in warmer climates. *Solanum commersonii* Dun. (2n = 2x = 24) has been reported to carry resistance to bacterial wilt caused by Rs (Laferriere et al. 1999; Fock et al. 2000; Aversano et al. 2015). Rs, a quarantine organism worldwide (A2 list, European and Mediterranean Plant Protection Organization), is a β-proteobacterium that infects the plant by colonizing the xylem. The infection blocks the flow of water and soluble nutrients, including organic ions, from the roots to the rest of the plant, causing wilting and subsequent death of the plant leading to high economic yield losses (Champoiseau et al. 2009; Albuquerque et al. 2015; Clarke et al. 2015). The pathogen is transmitted from soil, water or infected plant material into the plant through root invasion (Zuluaga et al. 2015). Bacterial wilt has been reported in tropical and Mediterranean climatic regions; however, this disease has also been recorded in temperate regions of the world. In the United States, Rs is classified as a select agent since it poses a severe threat to plant health and has zero-tolerance reinforcement quarantine regulations and sanitation protocols to avert the introduction of the pathogen (Champoiseau et al. 2009). Rs is among the key challenges facing seed and commercial potato production in tropical, subtropical and warm temperate regions of the world (Champoiseau et al. 2009).

The Rs genome harbors many transposable elements that contribute to genomic variation (Álvarez et al. 2010). This variation contributes to its ability to effectively colonize plant tissues and cause bacterial wilt (Álvarez et al. 2010; Clarke et al. 2015) and due to the variation within Rs groups, they are collectively referred to as a "species complex" (Fegan and Prior 2005; Champoiseau et al. 2009). Four comprehensive phylotypes of Rs, corresponding to geographical and genomic diversity groups have been described. The phylotypes can be identified with DNA primers specific to the complex (Carputo et al. 2009; Albuquerque et al. 2015). The phylotypes are further subdivided into sequevars based on the sequence of the endoglucanase (*egl*) gene (Champoiseau et al. 2009). Rs strains described as Phylotype I include lowland tropical strains and possess a wide host range. Phylotype II is known to mostly infect highlands potato growing regions and cold tolerant potatoes. It is also known as the brown rot strain which belongs to the Phylotype IIB – 1 (Franc et al. 2001; Cellier and Prior 2010). Phylotype IIA is documented to have a broad host range and has been predominantly isolated from tropical regions (Cellier and Prior 2010). The Phylotype III strains originated from Africa and Phylotype IV strains are from Indonesia (Champoiseau et al. 2009).

Diploid wild relatives of cultivated potato have been hybridized to *S. tuberosum* to access genes for disease resistance and stress tolerance (Jansky and Peloquin 2006). Conventional breeding strategies, coupled with disease testing, show potential to breed bacterial wilt resistant cultivars. The current research was conducted using clones from the wild potato species *S. commersonii*. Selfed progeny of *S. commersonii* were assessed for Rs resistance. This research assessed the wilting degree by artificially inoculating greenhouse-grown clones. The disease assessment identified the most promising clones to be used to study the genetic basis of Rs resistance. These resistant clones also are a source of Rs resistance that can be used in potato breeding.

Materials and Methods

Plant Material

S. commersonii is a diploid wild relative of the cultivated potato native to Uruguay, Argentina and Brazil with reported resistance to Rs, *Verticillium* wilt (*Verticillium dahlia*), potato tuber nematode (*Ditylenchus destructor*), early blight (*Alternaria solani*) and tolerance to low temperatures (Laferriere et al. 1999; Caruso et al. 2008). *S. commersonii* clonal seedlings were provided by Dr. John Bamberg (NRSP-6 Potato Gene Bank, USDA-ARS). Self-pollinations were carried out in the greenhouse using four CEC03 individuals selected from PI320266, which were previously reported to have bacterial wilt resistance. This self-fertilization of four different CEC03 individuals generated four populations. Based on SNP genotyping, the progenies were determined to only have alleles from *S. commersonii* (data not shown). One seedling was selected from each of the four self-fertilized populations to screen against two strains of the Rs pathogen. *S. commersonii* clones CEC03–03 and CEC03–08 and four *S. commersonii* progeny (MSEE908–03, MSEE912–08, MSEE914–06, MSEE915–02) and *S. chacoense*-derived M6, a susceptible check, were screened.

The seeds from the self-fertilized *S. commersonii* clones were planted and germinated in the tissue culture laboratory using Murashige and Skoog (MS) medium containing 3% sucrose 0.8% agar and pH of medium was adjusted to 5.8 (Murashige and Skoog 1962). The plantlets were kept in the growth chambers at 20 ± 2 °C under 16/8 h light/dark photoperiod. The tissue culture-propagated seedlings were transplanted in the greenhouse for rapid multiplication. One-and-half month old plant material were later transferred to growth chambers for the bacterial wilt resistance bioassay.

Bioassay for Rs Resistance

The Rs bacterial colony was grown for 48 h at 28 °C on Kelman's growth media having 2,3,5 triphenyl tetrazolium chloride (TTC) as previously described by Siri et al. (2009). Rs inoculum was prepared and diluted to1×10^7 cfu/ml. Rs screening was conducted at the University of Wisconsin, Madison. Two Rs strains were used in the study: the Rs strain UW551 (phylotype IIB sequevar 1) and CMR15 (phylotype I). Six-week-old greenhouse plants grown from tissue culture transplants were transferred to the growth chamber for inoculation. The growth chamber was maintained at 24–28 °C, with a 16/8 h light/dark photoperiod and 80% relative humidity. A Rs inoculum suspension with 0.9% saline solution was prepared and adjusted to a concentration of 1×10^7 cfu/ml. The roots were wounded to ensure entry of the pathogen prior to 50 ml of bacterial suspension being applied to the soil in the pots.

Disease Scoring After inoculation, each plant was assessed every day for 20 days for Rs disease progression and wilting symptoms. Symptoms were scored using a wilting degree (WD) rating in leaves according to Carputo et al. (2009) and Fock et al. (2001) where 0 = no wilted leaves, 1 = 1–25% wilted leaves, 2 = 26–50% wilted leaves, 3 = 51–75% wilted leaves and 4 = 76–100% of leaves wilted. In addition to evaluating WD, the WD scores were converted to percentage equivalents for calculation of the area under the disease progress curve AUDPC (Simko and Piepho 2012) and the relative area under the disease progress curve (RAUDPC) was used to estimate the disease progression over time.

Experimental Design and Statistical Data Analysis

Eight replicate plants of each clone for each Rs strain were placed in a randomized complete block design (RCBD). WD scores, by Rs strain and days post inoculation, and overall RAUDPC values, by Rs strain, were used for a one-way analysis of variance using JMP Pro 15 (SAS Institute, Cary, NC). Means comparisons were done with Tukey's HSD ($\alpha = 0.05$).

Results

Rs Evaluation

After inoculation of Rs strain UW551, most of the clones did not show Rs infection symptoms at 5 days post inoculation (DPI). All the clones showed some level of infection starting at 10 DPI, with *S. commersonii* progeny MSEE912–08 showing the least wilting (Fig. 1). By 15 DPI, *S. commersonii clone* CEC03–08 was more infected than the susceptible check, *S. chacoense* M6. *S. commersonii* progeny MSEE914–06 and M6 were statistically equivalent to CEC03–08 in WD score (4 = completely wilted) at 20 DPI. Based upon WD scores at 20 DPI, the most bacterial wilt resistant clone was MSEE912–08 with an incidence score of 0.4 for the UW551 strain (Fig. 1). CEC03–08, MSEE908–03 and MSEE915–02 had higher WD scores than MSEE912–08 but less than M6, the susceptible check.

Similar to the UW551 bioassay, most of the clones did not show symptoms of infection by the Rs strain CMR15 at 5 DPI. Again, CEC03–08 was completely wilted by 15 DPI, as was MSEE914–06, however susceptible check M6 was not completely wilted at 20 DPI. By 20 DPI, the disease progression RAUDPC score (Fig. 2) and WD scores were lowest for MSEE908–03 and MSEE912–08 (1–1.3 WD) for the CMR15 infection (Fig. 1). Based upon disease progression (Fig. 2) and WD scores at 20 DPI, the most bacterial wilt resistant clone was MSEE912–08 with an incidence score of 0.4 for the UW551 strain and 1.3 for the CMR15 strain (Fig. 1).

Discussion

The results from this study suggest that *S. commersonii* can be a source of bacterial wilt resistance in potato. Considering both strains of Rs in this study, we were able to identify MSEE912–08 as the most wilt resistant clone in this study, and as a useful resource for further wilt resistance studies. Our results did not identify clones with immunity to Rs infection. The results indicated a quantitative disease resistance that is expressed as a reduction in wilt symptoms rather than a total absence of wilting (Poland et al. 2009). MSEE912–08 had significant resistance to wilting from Rs for the two different strains tested. Our experiment showed the first symptoms of disease wilting 5 DPI in some clones (Fig. 1), which is consistent to previous work done by Ishihara et al. (2012) in which they examined transcriptomic information on the early response of tomato against Rs at 3–7 DPI. The expression of Rs resistance is believed to be under polygenic control and this durability for resistance can be prolonged when properly managed (Zuluaga et al. 2015).

There are challenges in screening for Rs resistance. Resistance is quantitative and Rs bioassays have a high level of variability. Escapes could be interpreted as resistance. Our bioassays were based upon eight replications of five genotypes for each Rs strain. Some of the *S. commersonii* clones were as susceptible as M6. The evaluation of the population used Rs strain UW551, one of the most aggressive strains of Rs as well as another phylotype (CMR15). Screening was conducted under optimum conditions that favored the development of disease symptoms. With the genetic variability of Rs it would be useful to see how durable this resistance is to other strains. The level of wilting resistance in the clones

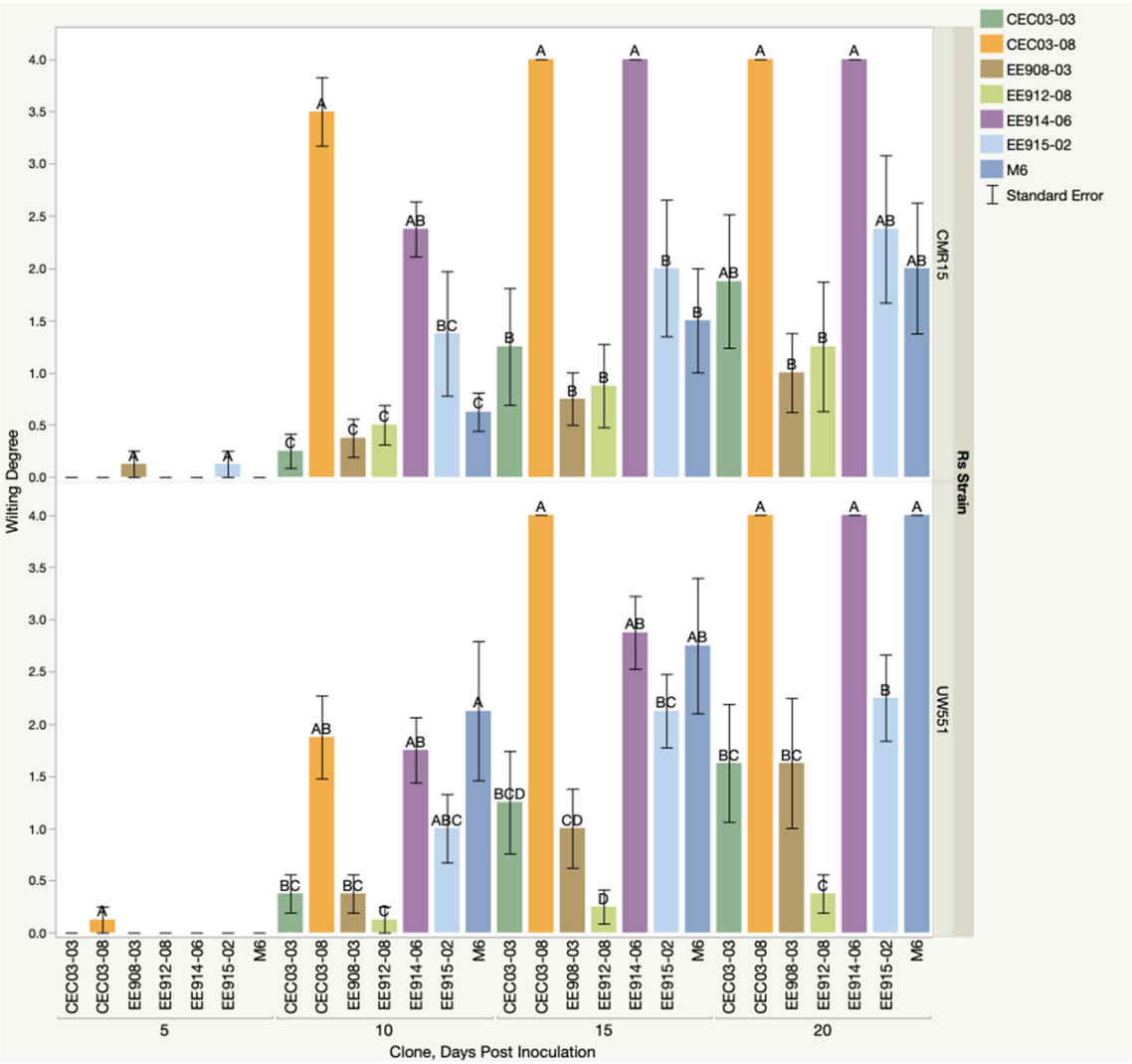

Fig. 1 *Ralstonia solanacearum* strains CMR15 and UW551 Wilting Degree ratings at 5, 10, 15 and 20-days post inoculation on *S. commersonii* clones, progenies and M6, susceptible check. Error bars indicate standard error. Means with the same letter designation, by DPI and Rs strain, are not significantly different as determined by Tukey's HSD ($\alpha = 0.05$)

observed in this experiment concurred with a previous study by Carputo et al. (2009). Our results showed resistant clones exhibited WD < 1. It would be valuable to know whether the genes segregating in this *S. commersonii* population coincide with those that Zuluaga et al. (2015) identified.

A study by Habe (2018) analyzed the inoculation age and determined that the resistance and susceptibility occurrence were comparable to the results in our study using six-week-old plantlets. The group analyzed nine different genotypes which were subjected to the same incubation temperature and the same strain (Phylotype I) of Rs. Siri et al. (2011) found that the Rs resistance to be unstable across different geographical regions. Therefore, geographically targeted breeding against the prevalent regionally specific strain should be considered to achieve meaningful progress in bacterial wilt breeding. In this study UW551 is similar to NAK66, an isolate from Kenya, our targeted breeding location.

S. commersonii, *S. brevidens* and *S cardiophylum* are among diploid species that have been utilized in Rs resistance and studies (Johnston and Hanneman 1980; Carputo et al. 1999) but crossing barriers have limited their use in resistance breeding. Previous research work used sexual hybrids of *S. multidissectum*, *S. sparsipillum*, and *S. chacoense* to transfer Rs resistance into a cultivated background. The resistance conferred was moderate resistance along with undesirable traits like high glycoalkaloid content (Siri et al. 2011). Fock et al. (2000) reported on bacterial wilt resistance derived from *S. phureja* was controlled by three unlinked genes. Furthermore, four major loci have equally been reported to play a key role in bacterial wilt in potato (Fock et al. 2001). There are reports that different sources of bacterial wilt resistance in solanaceous crops have different biological mechanisms, as different Rs strains have extensive genetic diversity (Huet 2014). Therefore, the best approach to identify sources

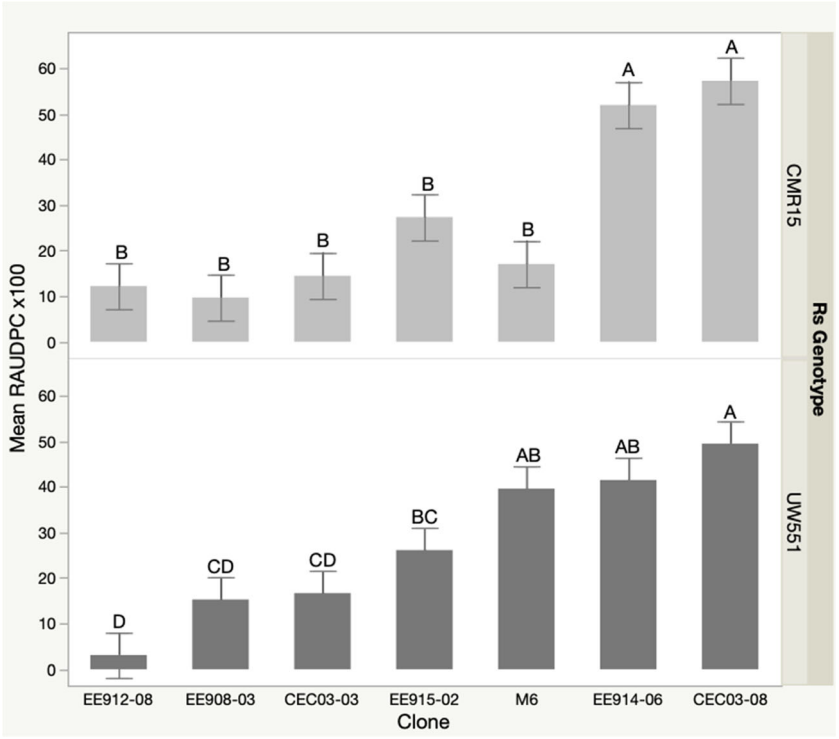

Fig. 2 RAUDPC ×100 values for 20 days post inoculation of on *S. commersonii* clones, progenies, and M6 (susceptible check) using *Ralstonia solanacearum* CMR15 and UW551 strains. Error bars indicate standard error. Means with the same letter designation, by Rs strain, are not significantly different as determined by Tukey's HSD ($\alpha = 0.05$)

of Rs resistance is by employing screening methods that employ aggressive strains and optimal pathogen conditions.

In order to achieve progress in diploid breeding, self-compatibility is critical to production of inbred lines. The discovery of a dominant S-locus inhibitor (*Sli*) gene has been critical for incorporating self-compatibility in diploid potato (Hosaka and Hanneman 1998). In developing germplasm for this study, we unexpectedly identified self-compatibility in this *S. commersonii* population. Further study of self-compatibility in this germplasm will inform integration of possible diverse sources of self-compatibility for use in diploid breeding. MSEE912–08 has been self-pollinated to create a mapping population to map Rs resistance in *S. commersonii*. Progeny will be screened in a greenhouse bioassay in Kenya using a local Rs strain NAK66. Once bacterial wilt resistance quantitative trait loci or genes are identified, they may be employed through marker assisted selection to support the development of improved cultivars with bacterial wilt resistance.

Resistant cultivars are part of an integrated disease management of Rs, however challenges confront bacterial wilt breeding such as genetic variability of the pathogen and combining desirable agronomic traits with effective levels of Rs resistance. When linkage drag occurs, unfavorable alleles from the unadapted donor species are transferred along with the target gene or QTL (Bernardo 2014). Additionally, durable resistance and adaptation to different agro-ecological zones must be considered in breeding efforts (Boschi et al. 2017). Continued research with *S. commersonii* can facilitate the introgression of bacterial wilt resistance and other desirable agronomic traits into diploid potato breeding programs.

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