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Research Application Summary

Understanding the virulence dynamics of bacterial wilt caused by *Ralstonia solanacearum* and the host plant defense responses

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Abstract

Bacteria wilt caused by *Ralstonia solanacearum* has continued to pose a significant threat to a wide range of agricultural crops. The direct and indirect damage caused by various *R. solanacearum* strains result in reduced yields and quality of crop produce, high costs of production and therefore loss of income, and this is a threat to food and nutritional security. Understanding the *R. solanacearum* virulence dynamics is essential for effective management of bacterial wilt. Nitric oxide (NO) is known as a key mediator of plant defense responses and functions as a signalling molecule against microbial pathogens. In this study, *R. solanacearum,* the causal agent of bacterial wilt disease in tomato, was shown to produce NO, which emanates via its denitrification pathway. The effects of NO on the host's defense response during tomato-*R. solanacearum* interactions was also investigated and bursts of NO at 6 and 14 h was observed in the tomato root tips of plants inoculated with wild-type *R. solanacearum*. However, high accumulation of NO was observed in bacterial deletion mutants ΔnorB and ΔhmpX mutants, while very little NO was detected with ΔaniA mutant. *R. solanacearum* produces much of NO which likely affects the host's defense response as shown also by eliminating NO from plants via inhibition of NO synthase in aniA mutant. NO-deficient ΔaniA mutant triggered lower expression of the tomato ethylene pathway defense gene PR1 and ACO5, while the NO-accumulating ΔnorB and ΔhmpX mutants triggered significantly higher expression. These results show that much NO is produced both by the plant and the pathogen during interactions and further suggests that the pathogen-produced NO plays a significant role in the induction of plant defenses. These insights could potentially be harnessed to come up with effective management systems for this devastating soil borne pathogen.

Key words: Deletion mutants, Pathogenesis, *R. solanacearum*, signalling, virulence

Resume

Le flétrissement bactérien causé par *Ralstonia solanacearum* continue de représenter une menace importante pour de nombreuses cultures agricoles. Les dommages directs et indirects causés par les différentes souches de *R. solanacearum* entraînent une baisse de rendement et de la qualité des produits agricoles, des coûts de production élevés et donc des

pertes de revenus, ce qui constitue une menace pour la sécurité alimentaire et nutritionnelle. La compréhension de la dynamique de virulence de *R. solanacearum* est essentielle pour une gestion efficace du flétrissement bactérien. L'oxyde nitrique (NO) est connu comme un médiateur clé des réponses de défense des plantes et fonctionne comme une molécule de signalisation contre les pathogènes microbiens. Dans cette étude, il a été démontré que *R. solanacearum*, l'agent causal du flétrissement bactérien de la tomate, produit du NO, qui émane via sa voie de dénitrification. Les effets du NO sur la réponse de défense de l'hôte pendant les interactions entre la tomate et *R. solanacearum* ont également été étudiés et des bouffées de NO à 6 et 14 heures ont été observées dans les extrémités des racines de tomates de plantes inoculées avec *R. solanacearum* de type sauvage. Cependant, une forte accumulation de NO a été observée dans les mutants de délétion bactérienne ΔnorB et ΔhmpX, tandis que très peu de NO a été détecté avec le mutant ΔaniA. *R. solanacearum* produit beaucoup de NO qui affecte probablement la réponse de défense de l'hôte comme le montre également l'élimination du NO des plantes via l'inhibition de la NO synthase chez le mutant aniA. Le mutant ΔaniA déficient en NO a déclenché une expression plus faible du gène de défense PR1 et ACO5 de la voie de l'éthylène de la tomate, tandis que les mutants ΔnorB et ΔhmpX accumulant du NO ont déclenché une expression significativement plus élevée. Ces résultats montrent que beaucoup de NO est produit à la fois par la plante et le pathogène pendant les interactions et suggèrent en outre que le NO produit par le pathogène joue un rôle important dans l'induction des défenses de la plante. Ces connaissances pourraient être exploitées pour mettre au point des systèmes de gestion efficaces contre cet agent pathogène dévastateur présent dans le sol.

Mots clés: Mutants de délétion, pathogénèse, *Ralstonia solanacearum*, signalisation, virulence

Introduction

NO is a highly diffusible and reactive gaseous molecule with a broad spectrum of functions in plants. Together with other reactive nitrogen intermediates including nitrate (NO_3), nitrite (NO_2) and nitrous oxide (N_2O), it regulates plant growth, (Besson-Bard *et al.,* 2008) and modulation of interactions in the environment. Additionally, NO is involved in plant defense signaling as shown in different plant species and organs in response to biotic stress during infection by microbes such as virus, bacteria and fungi (Zhou *et al.,* 2015). The interplay of enzymes involved in NO production in plants is poorly understood and is thought to be yet by unknown NOS-like enzymes (Fröhlich and Durner, 2011). NO biosynthesis in plants can occur through oxidative pathways including the nitrate reductase and mitochondrial or plasma membrane-associated. The reductive pathways include NO production from L-arginine or polyamine (Gupta *et al.,* 2011).

In plants, NO interacts with downstream signalling pathways such as JA and ethylene and mediating host defense response during microbial infection (Gupta *et al.,* 2011). The level of NO concentration highly determines plants' response and its central perception is known to occur via the identified group VII of Ethylene Response Factors (Gupta *et al.,* 2011). JA production is positively regulated by NO in response to *Pseudomonas syringae* pv. tomato (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2014). NO is shown to modulate the

activity of proteins through nitrosylation and tyrosine nitration (Besson-Bard *et al.,* 2008). In early symbiotic interactions, both plants and bacteria produce NO which is essential for establishment of the association as well as repressing the host defense reactions (Gupta *et al.,* 2011).

While the role of NO in plant-pathogen interactions has heavily been studied from the host perspective, recent studies highlight the role of the pathogen contribution. However, there is scarce knowledge on whether these pathogens produce NO and its role during the plant-microbe interactions. Some of these pathogens including bacteria that may have developed unique mechanisms of utilizing both the host-derived and their own NO either by detoxifying or converting the emanating signaling cascade into a physiological response (Arasimowicz-Jelonek and Floryszak Wieczorek, 2014). Interestingly, this topic has been limited to just a handful of fungal pathogens and the potential role of bacterial denitrification-produced NO has not been investigated. NO production in bacteria is mainly via the denitrification pathway during which NO_3- is reduced to NO_2- by the periplasmic nitrate reductase and NO_2- is reduced to NO by the respiratory nitrite reductase. NO is further converted into N_2O by NO reductase and eventually into N2 by N2O reductase or can be detoxified by a predicted flavohemoglobin into NO_3- aerobically (Arasimowicz-Jelonek and Floryszak Wieczorek, 2014).

Many bacterial plant pathogens have this pathway, but this study focused on denitrification of *Ralstonia solanacearum,* which causes lethal wilt disease in more than 200 plants species and is regarded as a species complex. It thrives in two separate ecological niches, inside the plant as a parasite and in the soil as a saprophyte (Genin, 2010). A hallmark of *R. solanacearum* virulence is the complex effector T3SS that the pathogen utilizes to attack and kill the host by shutting off the host defense response and poisoning them with nitric oxide (Genin and Denny, 2012). Moreover, *R. solanacearum* utilizes the EPS to avoid recognition, invades and proliferates into the host xylem vessels, an environment deficient of vital nutrients (Genin and Denny, 2012). At this point, its is known that NO production and consumption affects the pathogen but does this pathogen-produced NO level manipulation affects the host? Here this study measured NO production and defense gene expression in its natural host, tomato, in response to wild-type *R. solanacearum* and metabolic mutants that under- or over-produce NO. These experiments revealed that *R. solanacearum*-produced NO contributes greatly to the NO pool during the plant-pathogen interaction and that this NO plays a significant role in induction of plant defenses.

Materials and Methods

Plant material, growth conditions and NO treatment. To measure NO production during interactions between tomato and *R. solanacearum*, four day-old seedlings of wilt-susceptible tomato cultivar Bonny Best were used. Surface-sterilized seeds were grown on 1% agar supplemented with Hoagland's solution and incubated in the dark at 28^{o}C. *R. solanacearum* strains used in these experiments were grown in CPG medium (Hendrick and Sequeira, 1984) at 28^{o}C with antibiotics at concentrations of 15 g/ml gentamicin and 25 g/ml kanamycin when appropriate. All treatments were carried out in a 24-blackwell plate (Arctic White LLC, Bethlehem, Pennsylvania, USA) by submerging seedlings in a total volume of 2.5 ml per well. Seedlings were inoculated with the wild-type *R. solanacearum* tomato isolate strain GMI1000. Fluorescence microscopy was performed with variants of

GMI1000 expressing DsRed. To study the bacterial NO production, we used the GMI1000 deletion mutants ΔhmpX, ΔnorB and ΔaniA, which lack the indicated gene in the inorganic nitrogen pathway (Figure 1). Additionally, plants were exogenously treated with NO by adding 100nM of the NO donor spermine NONOate purchased from Cayman Chemical. Spermine NONOate was first dissolved in 0.01M NaOH and subsequent dilutions with sterile water to a final concentration of 30nM in the wells. To release NO, alkaline solution of spermine NONOate was added to 0.1M phosphate buffer (pH 7.4). Plants were mock-inoculated with sterile water as a negative control.

DAF-FM fluorescence and NO measurement. The NO-specific, non-cell permeable fluorescent dye DAF-FM (Life Technologies) was used to detect and quantify extracellular NO produced by tomato and bacteria. Plate wells containing 102 CFU/ml R. solanacearum, three tomato seedlings, both, or water alone were treated with 10 μM DAF-FM final concentration. 7mM DAF-FM stock solution was prepared by dissolving 1 mg in 350 ul of anhydrous dimethyl sulfoxide (DMSO) according the manufacturer's instructions. The plate was swirled gently and incubated statically for 5 min in the dark at room temperature. NO production was measured as relative fluorescence using a Bio Tek HT plate reader (Ex/Em: 485/528).

Construction of *R. solanacearum* mutant strains. Clean deletion mutants of *R. solanacearum* strain GMI 1000 used in these experiments were developed as previously (Dalsing *et al.,* 2015). Briefly, the GMI 1000 strain lacking the complete open reading frame (ORF) of ΔaniA, ΔnorB and ΔhmpX were generated via homologous recombination by way of targeted deletion constructs produced with splicing by overhang extension PCR (SOE PCR). The native promoter and the region of interest were amplified and inserted into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA). The region was then transferred to pRCT that does not replicate in *R. solanacearum* using Gateway technology and the complementing pRCT then moved into the corresponding mutant via natural transformation (Bertolla *et al.,* 1997; Dalsing *et al.,* 2015). Antibiotic resistance was used to select strains that had successful incorporation of the single copy of complementing genomic region into the selectively neutral attTn7 site and thereafter, PCR was conducted to confirm incorporation of the desired DNA fragment.

Gene expression analysis. Interactions between *R. solanacearum* and tomato plants is characterized by bursts of NO as observed. We hypothesized therefore that NO alters gene expression of both the host and the interacting partner. To study gene expression during tomato-*R. solanacearum* interactions, four-day old tomato seedlings were inoculated with the wild-type *R. solanacearum* strain GMI 1000 or mutants strains (ΔaniA, ΔhmpX, ΔnorB), treated with NO donors (200μm CysNO and 2.5mM sodium nitroprusside), NOS inhibitor L-NMMA or mock-inoculated with water as a control. Treatments were carried out in a black well plate as described earlier by submerging seedlings in a volume of 2.5 mL sterile water per well containing *R. solanacearum* suspension at 107CFU/mL and samples collected after 14 hpi. Three biological replicates each comprised of three seedlings were treated independently and picked by first rinsing three times in sterile water. An identical experiment was simulated in a greenhouse condition via soil-soak inoculation and RNA samples were picked from tomato midstems at the time of first symptom appearance at disease index one (DI=1).

The total RNA was extracted from seedlings roots and midstems using Direct-zolTM MiniPrep Kit (Zymol Research, U.S.A.) according to manufacturer's instructions, followed by DnaseI digestion with Turbo DNA-free Kit (Ambion, Austin, U.S.A.). RNA concentration and purity was determined via micro spectrometry using Nanodrop (Nanodrop, Thermo Fisher Scientific) and the quality further assessed via gel electrophoresis. For gene expression analysis in *R. solanacearum*, the same RNA sample from infected plants was used. First strand cDNA synthesis was performed with 0.1µg of total RNA as a template using the SuperScript III Reverse Transcriptase kit (Invitrogen) and 0.5 µl of gene specific primers for PR1, and ACO5 for bacteria according to manufacturer's protocol. Where necessary, quality and absence of genomic DNA contamination was checked by semi-quantitative RT-PCR with the house-keeping reference genes actin and SerC for plant and bacteria respectively. cDNA (2 µl) was used for RT-qPCR to determine differences in expression of these genes in reference to the house-keeping genes actin and SerC (ref).

Reactions were performed in triplicates at 25µl with Power SYBR reagent (Applied Biosystems, Carlsbad, CA) using ABI PRISM 7300 real-time PCR system (Applied Biosystems). Thermo cycler conditions were 50^{o}C for 2 min, 95 ^{o}C for 10 min, 95 ^{o}C for 15s, annealing temperature for 15 s, 60 ^{o}C for 10min and plate read at 60 ^{o}C. The annealing temperatures for primers used ranged between 53, 56 and 60 ^{o}C. The relative quantification was carried as previously described (Jacobs *et al.,* 2012) and analysis using $\Delta\Delta C_T$ method according to Milling *et al.*
(2011). For each treatment, 3 biological replicates were conducted.

Bacteria inoculation and culture. *R. solanacearum* cells used for inoculation were prepared from overnight CPG by centrifugation at 6,000 rpm for 10 min and thereafter, resuspended to appropriate cell densities in sterile water. Bacteria cells resuspended at 102CFU/mL and 107CFU/mL were used for microscopy and NO measurements, respectively. 250 ul of 108CFU/mL bacteria suspension was loaded into each well to a final volume of 2.5 mL and density of 107CFU/mL. In soil soak inoculation, tomato plants (wilt susceptible genotype Bonny Best) grown in pots containing 80 g Sunshine Redi-earth professional plant growth mix (Sun Gro Horticulture, Agawarm, MA) and watered with Hoagland's solution. Three-weeks-old plants were soil soak inoculated as described previously (Dalsing *et al.,* 2015). Subsquently 50 mL of *R. solanacearum* prepared from overnight CPG was suspended in water to an OD600 of 0.1 with a final cell density of 5 x 107 CFU/g soil. Sampling of midstems was conducted when wilt symptoms appeared at DI=1.

Statistical analyses. Statistical analyses were performed using JMP version 12.0.1 (SAS institute). Data for NO measurements and fluorescence quantification were subjected to ANOVA and the significance differences between individual means was determined using Tukey`s pairwise comparison test and Student t at 5% confidence level.

Results and Discussion

Nitric oxide is produced when *R. solanacearum* interacts with tomato plants. To determine if and when nitric oxide (NO) is present in *R. solanacearum*-tomato plant interactions, we measured NO in suspensions of the interacting partners over 14 hours

using the molecular probe DAF-FM. Significantly more NO was produced during the *R. solanacearum*-tomato interaction than by either *R. solanacearum* (WT-wildtype) or tomato (TM) plants alone (Figure 1). Interestingly, the amount of NO present in the co-suspension was greater than the additive NO produced by the bacteria and tomato plants alone. This indicates that while both partners produce NO under these conditions, the interaction between the two results in additional production of NO.

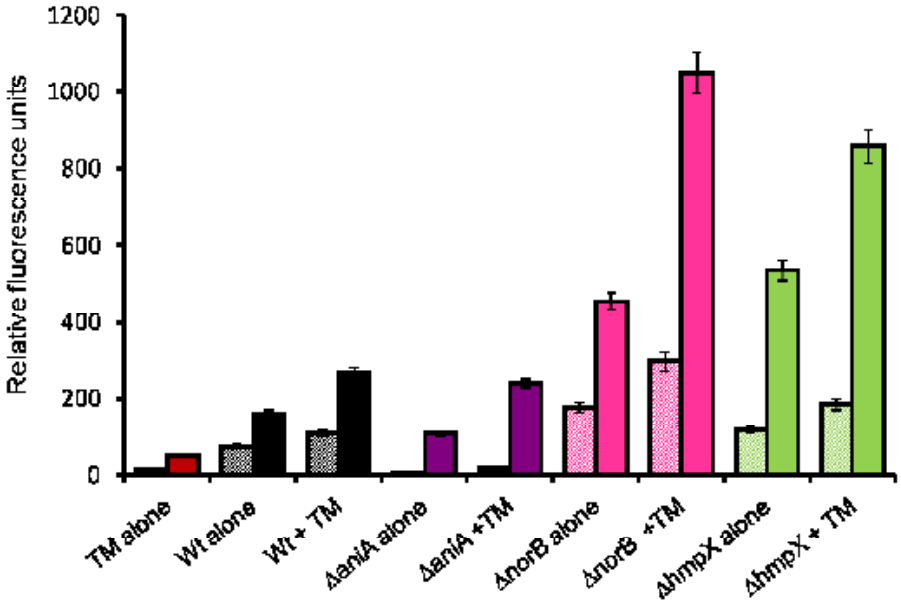

Figure 1. NO production (measured with DAF-FM) by tomato seedlings and *R. Solanacearum* wildtype and mutants

R. solanacearum contributes to the nitric oxide pool during its interaction with tomato plant. Relative levels of NO was quantified after 14 hours co-incubation of tomato plants with several *R. solanacearum* strains (Figure 1). As described earlier, ΔaniA *R. solanacearum* lacks a nitrite reductase that is essential for NO production. Hence, any NO present in the ΔaniA strain-tomato interaction is mainly plant-produced. We observed that during this interaction, six (6) hours post inoculation, no NO was detectable. After 14 hours, NO levels were similar to during wild-type *R. solanacearum*-tomato interactions. These results suggest that *R. solanacearum* production of NO influences the total NO concentration in the interaction and the kinetics of its production by the plant.

Both ΔnorB and ΔhmpX *R. solanacearum* strains lack the full ability to metabolize NO and, thus, both of these strains tend to over-accumulate NO. We observed that during both ΔnorB and ΔhmpX *R. solanacearum*-plant interactions, significantly more NO was detected than in wild-type interactions. This suggests again that the pathogen's NO production affects the total NO concentration in the interaction. Additionally, the plant likely does not tightly control NO levels during *R. solanacearum* infection and at least a portion of this NO is *R. solanacearum*-produced.

Pathogen-produced nitric oxide influences tomato defense gene expression. NO was shown to affect tomato defense gene induction as demonstrated by our findings. *R. solanacearum* and tomato plants produces NO and these interactions led us to hypothesize that pathogen NO metabolism may influence host defense responses. To determine whether *R. solanacearum* NO metabolism affects tomato defense gene expression, RT-qPCR was performed on tomato seedling roots following 14 hours of incubation with wild-type, ΔaniA, and ΔnorB *R. solanacearum*. Compared with the water-inoculated control, wild-type *R. solanacearum* induced PR1 and ACO5 gene expression, indicators of salicylic acid and ethylene production, respectively. The ΔaniA mutant induced much less defense gene expression while the ΔnorB strain induced much more gene expression in both the salicylic acid and ethylene pathways (Figure 2).

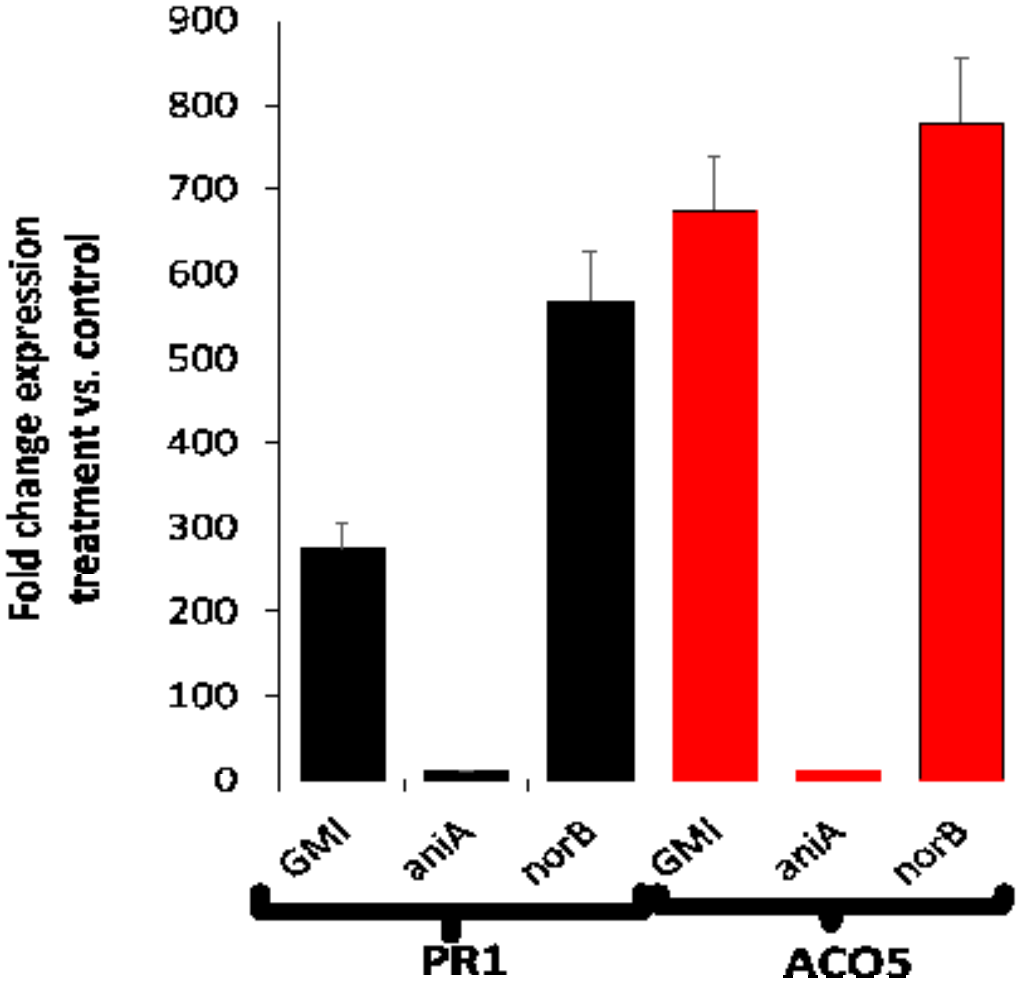

Figure 2. Expression of tomato NO-induced defense genes PR1 and ACO5, indicators of salicylic acid and ethylene production pathways

Conclusion

NO is produced by the host plant for defense against the pathogen during interactions. *R. solanacearum,* a soilborne vascular pathogen, triggers production of NO in plants during infection and by itself also, produces significant amounts of NO. As a signalling molecule, NO plays a key role in induction of plant defenses as demonstrated by significant expression of genes PR1 and ACO5 in this study, an aspect that is essential for plant protection. However, *R. solanacearum* can also utilize NO to promote its virulence adding to the dynamic nature of N. The essential nutritive role of nitrate in plants cannot also be overlooked and this diverse knowledge is essential in the development of management system for bacterial wilt in food crops.

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References

Arasimowicz-Jelonek, M. and Floryszak-Wieczorek, J. 2014. Nitric oxide: An effective weapon of the plant or the pathogen? *Molecular Plant Pathology* 15: 406–416.

Besson-Bard. A., Pugin, A. and Wendehenne, D. 2008. New insights into nitric oxide signaling in plants. *Annual Review of Plant Biology* 59: 21–39.

Dalsing, B. L. and Allen, C. 2015. Nitrate assimilation contributes to Ralstonia solanacearum root attachment, stem colonization, and virulence. *Journal of Bacteriology* 196: 949–60.

Fröhlich, A. and Durner, J. 2011. The hunt for plant nitric oxide synthase (NOS): Is one really needed? *Plant Science* 181: 401–404.

Gavet, O. and Pines, J. 2010. Progressive Activation of CyclinB1-Cdk1 Coordinates Entry to Mitosis. *Developmental Cell* 18: 533–543.

Genin, S. and Denny, T. P. 2012. Pathogenomics of the *Ralstonia solanacearum* species complex. *Annual Review of Phytopathology* 50: 67–89.

Gupta, K.J., Fernie, A.R., Kaiser, W. M., van Dongen, J. T. 2011. On the origins of nitric oxide. *Trends in Plant Science* 16: 160–8.

Hendrick, C. and Sequeira, L. 1984. Lipopolysaccharide-Defective Mutants of the Wilt Pathogen *Pseudomonas solanacearum*. *Applied and Environmental Microbiology* 48: 94–101.

Jacobs, J. M., Babujee, L., Meng, F., Milling, A. and Allen, C. 2012. The in planta transcriptome of *Ralstonia solanacearum*: conserved physiological and virulence strategies during bacterial wilt of tomato. *MBio* 3 (4):e00114-12.

Milling, A., Babujee, L. and Allen, C. 2011. *Ralstonia solanacearum* extracellular polysaccharide is a specific elicitor of defense responses in wilt-resistant tomato plants. *PloS one* 6: e15853.