ABSTRACT

TSENG, HSIEN-TZER. Effects of Three Chemical Compounds on *Ralstonia solanacearum* Physiological Functions and Disease Development. (Under the direction of Dr. David F. Ritchie)

Bacterial wilt caused by *Ralstonia solanacearum* is an important soil-borne disease of many economically important crops in tropical and subtropical regions of the world. *Ralstonia solanacearum* is a gram-negative, aerobic rod in the β-subdivision of Proteobacteria and has been the subject of numerous studies on host-pathogen interactions.

Three chemical compounds known to have anti-microbial and anti-biofilm activities were examined: 3-indolyacetonitrile (IAN), *p*-benzoquinone (pBQ), and 6,7-dihydroxycoumarin (6-7-D). The minimal inhibition concentration against Rs for each compound was 80 μM, 25 μM, and 125 μM for IAN, pBQ and 6-7-D, respectively. Sub-inhibitory concentrations, 20 μM for IAN, 12.5 μM for pBQ, and 50 μM for 6-7-D, were used to determine bacterial growth in broth culture. IAN was significantly more effective in inhibiting bacterial growth compared to pBQ and 6-7-D. There was no difference in colony morphology when Rs was grown on media amended with sub-inhibitory concentrations of each of the three compounds. Biofilm formation was enhanced when Rs was treated with IAN and 6-7-D. Fluorescence microscopy with GFP-labeled W7 strain demonstrated that IAN resulted in greatest reduction of bacterial colonization of plant roots. In laboratory experiments, tobacco cultivars K326 and Speight168 were treated with the compounds 48-hours before, at the same time or 48-hours after inoculation. IAN was most effective in limiting disease incidence among the three tested compounds, and treating at the time plants were inoculated offered the most suppression regardless of the compound. In greenhouse experiments, disease progression was slower in plants treated with IAN and pBQ than the untreated or plants treated with 6-7-D for both K326 and Speight168.

At a concentration without affecting bacterial growth, IAN was the most effective compound in limiting bacterial flagellar motility, metabolism of various carbon sources, and disease severity, yet increased the production of extracellular polysaccharide (EPS) in culture. Quantitative real-time PCR revealed that expression of crucial EPS regulators, PhcA and XpsR, concurred with the biochemical assay results. To a lesser degree, 6-7-D also inhibited flagellar motility and increased EPS production in culture but did not inhibit *R. solanacearum*'s ability to metabolize different carbon sources. pBQ was least effective in interfering with the virulence factors and metabolism. These results provided insight on how chemical compounds may be affecting multiple aspects of infection and disease development processes. Utilizing the knowledge of bacterial physiology and pathogenicity can provide researchers with valuable criteria for evaluating potential chemical agents, instead of simply looking for antimicrobial compounds based on growth inhibition.

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Effects of Three Chemical Compounds on *Ralstonia solanacearum* Physiological Functions and Disease Development

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DEDICATION

I dedicate this work to my family, fiancée, friends, and cat. I would not be where I am today without your constant love, encouragement, and support.

BIOGRAPHY

Hsien-Tzer Tseng, a native of Taiwan, raised by his mother Ling and father Nien-San. His interests in science began at a young age in his first biology classes. He earned his B.S. at the National Chung Hsing University, Taichung, Taiwan, in 2007. He completed his M.S. degree at the University of Georgia under Dr. T. Denny's supervision, investigating the type II secretion system of *Ralstonia solanacearum*. He was awarded the Cedric Kuhn Award for Outstanding M.S. student in Plant Pathology from the Georgia Association of Plant Pathologists. He joined Dr. A. Mila's lab at North Carolina State University as a research associate in 2010. In this capacity he broadened his knowledge in extension services and connecting lab with field experiments. After his brief time away from science due to the commitment to his nation, he came back to North Carolina State University in 2014 to work on his Ph.D. in the same lab, investigating the effect of chemical compounds on *R. solanacearum* physiology and their potential application as management tools.

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CHAPTER 1

Review of the Literature

Introduction

Bacterial wilt (BW) caused by *Ralstonia solanacearum* is an important soil-borne disease of many economically important crops in tropical and subtropical regions of the world. *Ralstonia solanacearum* is a gram-negative, aerobic rod in the β-subdivision of Proteobacteria (Stackebrandt et al. 1988) and has been the subject of numerous studies on host-pathogen interactions. The organism is one of the world's most important phytopathogenic bacteria because of its lethality, persistence, wide host range, and broad geographic distribution (Denny 2006). The pathogen enters plant roots via wounds or where secondary roots emerge, invades xylem vessels and rapidly spreads throughout the vascular system. The bacterial population within the vascular system can reach greater than 10^9 colony-forming units (CFU) $gram^{-1}$ fresh weight, producing large amounts of plant-cell-wall-degrading enzymes and high-molecular-mass extracellular polysaccharide, which eventually lead to vascular dysfunction and cause the plant to wilt and die (Schell 2000).

Bacterial wilt of tomato, pepper, eggplant, and Irish potato caused by *R. solanacearum* were among the first diseases that E. F. Smith proved to be caused by a bacterial pathogen (Smith 1920). The disease remains one of the most intractable plant diseases known. To reduce the yield losses due to BW, an integrated disease management program is required (Saddler 2005). Such a program includes the use of pathogen-free planting material, planting resistant cultivars, rotating susceptible crops with non-host crops, and use of a fumigant (Fortnum and Martin 1998; Yuliar and Toyota 2015). However, the wide host range of *R. solanacearum* limits rotational options. In severely infested fields, an effective rotation could require multiple years out of high value host

crops (Kelman, 1998). The use of resistant cultivars is one of the most cost-effective strategies for managing plant diseases, but resistance to BW in tomato and tobacco is quantitative and strongly affected by environmental conditions such as soil temperature, pH, and moisture (Scott et al. 2005; Bittner et al. 2016). Although soil fumigants provide good control in a given year, they have been reported to have limited success in the long run because of the vertical movement of *R. solanacearum* in the soil that may allow the bacterium to recolonize fumigated beds quickly (Driver and Louws 2002; Satou et al. 2006). Furthermore, the use of fumigants is in decline due to restrictions imposed by Environmental Protection Agency and other international organizations.

This review discusses the current understanding of the disease cycle, important virulence factors in disease development and current management strategies. By analyzing the effects of selected chemical compounds on the pathogen's physiology and disease development processes, it is anticipated that novel approaches to enhance the management of this disease may be discovered.

Disease cycle

Susceptible hosts infected by BW may contain 10^8 to 10^{10} cfu/g tissue of R. solanacearum and they usually die from the disease. The large number of bacterial cells shed from roots of infected plants (Elphinstone 1996) and bacterial ooze on plant surfaces (Buddenhagen and Kelman 1964; Kelman 1953) enter the surrounding soil or water and contaminate farming equipment. Because of the soil's complex environment, survival of the bacteria in soil is not well understood. Soil moisture and temperature are the main factors affecting the survivability of *R. solanacearum*. Other factors may include the soil type, soil depth, host plant debris, organic

matter content, nutrient level, and soil microbiome (Coutinho 2005). High amounts of organic material often correlates with decreased pathogen survival (Hayward 1991). In the absence of a true host, the pathogen must colonize the more nutrient rich soil near roots or latently infect roots of plants that remain asymptomatic for long term survival (> 2 years) (Coutinho 2005; Elphinstone 1996; Janse et al. 2004). Contaminated irrigation water also serves as an important route for pathogen dispersal and inoculation (Elphinstone 1996). *R. solanacearum* can survive in water from weeks to years depending on the abiotic and biotic factors.

To initiate the infection, *R. solanacearum* must be able to locate and approach a host's rhizosphere and invade an opening on host root that exposes internal tissue (Tans-Kersten et al. 2001; Yao and Allen 2006; Buddenhagen and Kelman 1964). Such openings may be caused by nematodes, farming equipment, or occur during normal plant development. For example, the development of lateral roots requires meristem cells growing outward from the central cylinder and breaking through the endodermis, root cortex, and epidermis (Peterson et al. 1981). For tomato plants, this site can be invaded by *R. solanacearum* (Vasse et al. 1995). To access these openings, the pathogen requires flagellar-mediated swimming motility and chemotaxis attraction to root exudates (Tans-Kersten et al. 2004; Yao and Allen 2006).

After invading a susceptible host, *R. solanacearum* cells multiply and move systemically within the plant. As the bacterial population increases in the nutrient-poor xylem, wilting symptoms occur after extensive pathogen colonization. Few studies have examined the systemic colonization by the pathogen histologically, and most were in tomato plants (Vasse et al. 1995; Vasse et al. 2005; Denny 2006). In general, bacteria were observed in the central cylinder of lateral roots within the first day or two after inoculation. The bacteria then progress into xylem vessels in the taproots and lower stem. Similar results were observed on susceptible tobacco

cultivar K326 (Bittner et al. 2016). In most plants, the end walls of functional vessels are completely degraded, resulting in few physical barriers to block axial spread of the bacteria once it has invaded xylem vessels. During the process of bacterial colonization within host xylem, development of tylosese may occur from adjacent parenchyma cells. In susceptible tomato plants, tylose formation is usually too infrequent or too slow to prevent pathogen migration and may instead lead to additional disruption of uncolonized vessels (Nakaho et al. 2000). A recent study has also demonstrated the importance of bacterial biofilm development and the modulation of biofilm structure in host xylem as being important for systemic spread and virulence (Tran et al. 2016). The pathogen tends to congregate near the xylem vessel pits often with adjacent parenchyma cell death. The pit membranes are broken down, likely by cell-wall-degrading enzymes (CWDEs) secreted by the bacteria, allowing the pathogen to spread into adjacent vessels or the space occupied by dead parenchyma cells (Grimault el al. 1994; Nakaho et al. 2000).

As pathogen density increases throughout the infected plant, wilting symptoms begin to appear. In root-inoculated tomato, wilting begins as bacterial density reaches 4×10^7 cfu/g tissue at the midstem (McGarvey et al. 1999). At this stage of disease development, extracellular polysaccharide (EPS) produced by the bacteria accumulate to about 10 µg/g tissue in the taproot, hypocotyl, and midstem. In fully wilted plants, EPS may accumulate over 100 µg/g tissue (McGarvey et al. 1999). It is believed that the high bacterial cell density and EPS, along with byproducts of plant cell wall degradation and plant-produced tyloses all contribute to the wilting of host plant.

Virulence factors

Ralstonia solanacearum occupies two distinctively different environmental niches: soil and host plant xylem. The bacterium can survive in soil or water as a saprophyte for years (Hayward 1991). When a susceptible host plant is present, the bacterium enters the host through wounds, root elongation zones, or site of lateral root emergence and colonizes the root cortex to invade xylem vessels (Denny 2006; Hayward 1991). Virulence gene expression is important for the *R. solanacearum* infection and colonization processes.

Phenotype conversion (PhcA) regulon

Ralstonia solanacearum colonizes two distinct environmental niches: soil environment where competition of nutrients is intense, and the favorable environment inside a host plant. In order to cope with the varied environments, the amount and type of bacterial gene products undergo a great deal of change. This change of gene expression pattern in *R. solanacearum* has been described as "phenotype conversion" (Brumbley et al. 1993). The Phc (phenotype conversion) system, composed of PhcA, a LysR-type transcriptional regulator, and the products of *phcBSRQ* operon, is the core of the complex network of virulence and pathogenic gene regulation in *R. solanacearum*. While characterizing the *phc* operon, researchers found that inactivating *phcB* would result in the same phenotype as inactivating *phcA*, and the *phcB* mutant can be restored to wild-type by exposing the mutant to bacterial culture supernatants (or vapors) from wild-type cells (Clough et al. 1994). The active compound responsible for complementing the *phcB* mutant was purified from culture supernatants and identified as 3-OH palmitic acid methyl ester (3-OH PAME) (Flavier et al. 1997). The recognition of 3-OH PAME by *R. solanacearum* is highly specific, minor alteration in the acyl chain or methylester reduces its activity greatly, and the expression of the Phc regulon can be activated by 3-OH PAME at

concentrations as low as 5nM (Flavier et al. 1997). Recent genome sequence analysis indicated that production and sensing of 3-OH PAME is restricted to three genera of the β-proteobacterium Burkholderiaceae family, including all sequenced *R. solanacearum* strains, *R. syzygii*, *R. picketii*, three *Cupriavidus* species, and some *Burkholderia* species (Guidot et al. 2007; Remenant et al. 2010, 2011).

In summary, PhcB is responsible for synthesizing 3-OH PAME, which accumulates in the extracellular environment as bacterial cell density increases. The two-component regulatory system PhcS/PhcR responds to the threshold concentration of 3-OH PAME and increases the level of functional PhcA. PhcA then activates the production of multiple virulence factors while suppressing the survival and invasion related functions of the bacteria (Denny 2006).

Some of the best studied traits involved with *R. solanacearum* virulence that are regulated directly or indirectly by PhcA include EPS production, motility, CWDEs production, and expression of effector proteins that are secreted by the type III secretion system (Genin and Denny, 2012).

Flagellar Motility

To initiate the infection, *R. solanacearum* must come in contact with the host rhizosphere. *Ralstonia solanacearum* has 1 to 6 polar flagella to mediate its swimming motility. Mutation in the flagellin gene (*fliC*) or flagellar motor protein (*fliM*) prevents motility on soft agar plate (Tans-Kersten et al. 2001). In addition, non-motile mutants have significantly reduced ability to cause disease on tomato when inoculated through biologically representative soil drench methods. However, when *fliC* mutant was inoculated directly on cut tomato petioles, which bypasses entering host plants from soil, disease severity was comparable to wilt-type *R. solanacearum* (Tans-Kersten et al. 2001). This result suggests that the pathogen only requires

flagellar motility at the initial infection stage. Once the bacteria successfully enter the host, flagellar motility is not required. This corresponds to the finding that *R. solanacearum* isolated from host xylem vessels are mostly non-motile (Tans-Kersten et al. 2001).

Early research had demonstrated that the pathogen frequently becomes nonmucoid and avirulent in culture (Smith 1896). The spontaneous mutants which lack extracellular polysaccharide (EPS) production, have increased motility and aerotaxis, while wild-type isolates which produces large amount of EPS, were mainly non-flagellated or non-motile when grown on rich solid media or broth for 24 to 48 h (Kelman and Hruschka 1972). Research has shown that in *R. solanacearum* regulation of motility is linked with bacterial cell density and other virulence factors (Allen et al. 1997; Brumbley et al. 1993; Schell 2000). Swimming motility has been shown to be regulated by three regulatory cascades: the master regulator of motility FlhDC, (Tans-Kersten et al. 2004) a two-component regulator PehSR (Allen et al. 1997), and the quorum sensing (QS)-responsive global virulence gene regulator PhcA (Brumbley et al. 1993; Clough et al. 1997).

Previous research has demonstrated that mutations in *flhDC* and *pehSR* would both cause the bacteria to become non-motile. In addition, when *flhDC* promoter region was inserted upstream of a gus (*uidA*) reporter gene plasmid construct, no detectable *flhDC::gus* expression was found in *pehSR* mutant background (Allen et al. 1997). This result indicates that PehRS modulates expression of motility via the transcription of *flhDC*. Inactivation of the Phc system causes *R. solanacearum* to become highly motile even at high cell density, indicating that PhcA negatively regulates motility, and is consistent with the observation that *R. solanacearum* cells taken from xylem fluid, where cells are crowded (>$5x10^8$ cellls/ml), are mostly non-motile (Clough et al. 1997). In is known that QS affects *R. solanacearum* flagellar motility. However,

when bacteria are non-motile and multiplying, they will congregate into microcolonies, but previous mutation studies did not characterize how non-motile cells behave in terms of QS-related gene expressions.

Chemotaxis

The chemotaxis behavior of *R. solanacearum* has been described by Yao and Allen (2006). The *R. solanacearum* strain K60 was attracted to various chemicals, plant root exudates, and to plant roots themselves. The mechanism of signal transduction and response regulation for chemotaxis has been well studied in *Escherichia coli* (Stock and Surette 1996). Briefly, cell membrane-associated receptors detect environmental stimuli and respond by changing their conformation. This change triggers autophosphorylation of the cytoplasmic histidine autokinase CheA, which forms a complex with the receptor through a coupling protein called CheW. CheA then transfers its phosphate group to the response regulator, CheY. The phosphoryl-CheY then interacts with the flagellar motor to switch its direction of rotation and therefore alters the bacterial tumbling rate. The net effect of these rate changes is movement toward favorable conditions or away from unfavorable ones. In *R. solanacearum*, motile but non-tactic mutants lacking CheA or CheW were less virulent compared to wild-type and were similarly less virulent to non-motile *fliC* mutant (Yao and Allen 2006).

Extracellular polysaccharide

Extracellular polysaccharide (EPS), a long polymer with a repeating unit of N-acetylated monosaccharides, is a major virulence factor for *R. solanacearum*. EPS is required for *R. solanacearum* to cause wilting and killing the host plants (Denny and Baek 1991; Saile et al 1997). The exact biological function of EPS for *R. solanacearum* pathogenicity is not clear. It is believed that the large amount of accumulated EPS in host xylem plugs the xylem vessels and

contributes to the wilting symptoms. In resistant tomato cultivar, Hawaii 7996, a mutant lacking EPS production induced significantly less defense responses when compared to wilt-type strain. In susceptible tomato cultivar Bonny Best, wild-type strain and EPS mutant did not elicit significant different level of defense response (Milling et al. 2011). This result suggests that EPS does not function as a covering for the bacteria when plants try to recognize bacterial surface structures. EPS is associated with development of biofilm as the main part of the biofilm matrix. It provides mechanical stability of biofilms and assist the bacterial cells attachment to surface (Flemming and Wingender 2010). EPS also offers a protection layer from environmental stress and antimicrobial agents (Limoli et al. 2015). Recent research has demonstrated that *R. solanacearum* indeed develops biofilm in host xylem and intercellular spaces (Mori et al. 2016; Tran et al. 2016). In addition to forming biofilm, the fine-tuning of the EPS and other components of the biofilm matrix is also important for *R. solanacearum* dispersal in the xylem vessels (Tran et al. 2016).

Proteins involved in EPS biosynthesis pathway are encoded by the 16-kb *eps* operon (McGarvey et al. 1998). High level of EPS production requires the function of multiple regulatory systems, including the QS Phc system, two sets of two-component systems *vsrA*/*vsrD* and *vsrB/vsrC*, and *xpsR* (Schell 2000). XpsR functions as a transcriptional activator that binds to the promoter region of the *eps* operon (Huang et al. 1998). The signals for the two-component systems are unknown, but active response regulators from both systems (VsrD and VsrC) are required for the optimal expression of EPS and mutations in the two-component systems reduce transcription of *xpsR* and *eps* (Huang et al. 1998). Instead of activating *eps* promoter directly, PhcA binds to the promoter of *xpsR*. Being part of the PhcA regulatory system means that EPS regulation responds to cell density. More importantly, researches have demonstrated that the

quorum-sensing response is similar both in culture and in host stem (Kang et al. 1999; Jacob et al. 2012).

Disease management

Management of bacterial wilt requires a comprehensive approach, often referred to as Integrated Disease Management (IDM). Relying solely on a single strategy cannot reduce the incidence or severity of BW in regions where the bacteria is endemic (Saddler, 2005). A successful IDM program requires the use of pathogen-free planting material, planting less susceptible host cultivars, and crop rotation scheme with non-host or resistant crops (Akiew and Trevorrow, 1994; Saddler, 2005). Aside from soil fumigation with chloropicrin, chemical application options are limited for BW management. Studies have examined the potential biological control agents, but their effectiveness was often inconsistent.

Cultural practices

Avoid introducing the pathogen by using pathogen-free planting material and irrigation water is the first step of management. However, in the southeastern United States, the pathogen strain affecting tomato and tobacco is already well established in the soil (Hong et al. 2012). Crop rotation is the best cultural control strategy for areas where *R. solanacearum* is already endemic. Eliminating volunteer plants and weeds that promote survival of the pathogen is an important component of successful rotation. Several grasses have been demonstrated to be effective in reducing BW incidence (Akiew et al. 1994). In North Carolina, a two to three-year rotation with fescue, small grains, or soybeans is generally recommended for tobacco production to reduce the risk of BW (Thiessen 2019). Sweet potato had also been tested as a good rotation crop in Africa (Lemage et al. 2005). However, the pressures to produce a high cash value crop and limited land availability are some of the problems for applying crop rotation.

Earlier research has attempted to manage bacterial wilt with various soil amendments. For example, S-H mixture (Sun and Huang 1985) containing bagasse, rice husk powder, oyster shell powder, urea, etc. have been used to reduce disease incidence in naturally infested fields of tobacco and tomato (Yao et al. 1994). Interestingly, heat treating the amendment mixture prior to application reduces the efficacy of the mixture, suggesting that some biological element was involved with the mechanism. Application of stable bleaching power was tested in Nepal (Dhital et al. 1997) with disease suppression of 70-89% in field trials; similar experiments were conducted in India in conjunction with deep ploughing (Kishore et al. 1996). Combination of plant compost products and chemical fertilizers (NPK) have been reported to reduce disease intensity on banana (Roy et al. 1999). However, contrasting results would suggest that effects of these strategies can be soil dependent.

In Japan, India, and southeast Asia, grafting susceptible but horticulturally desirable scions of eggplant or tomato onto bacterial wilt resistant rootstocks have been applied since the early 1990s (Grimault and Prior 1994, Nakaho et al. 2000). Such an approach has been adopted for tomato production in the Southeastern U.S. as well (Rivard et al. 2012).

Host resistance

Currently, there is no gene-for-gene type qualitative resistance available for most crops susceptible to bacterial wilt. Available sources of resistance are usually polygenic. The only example of resistance with dominant resistance genes is in peanut (*Arachis hypogaea*) (Liao 2005; Jiang et al. 2017). In most solanaceous crops, tolerance to bacterial wilt has been achieved, and resistant varieties are useful as part of an integrated disease management package (Thoquet et al. 1996; Patil et al. 2012). Tolerance to the disease (i.e. satisfactory yield even with infection) indicates that when disease pressure is high or weather conditions are excessively hot or wet, the

crop may still suffer significant loss. Various programs around the world are still actively breeding for better resistant cultivars.

Chemical control

Commercial chemicals or antibiotics are not effective in controlling BW. Fumigation with chloropicrin is recommended in fields with high disease pressure (Thiessen 2019). Soil fumigation has little success against the bacterium on tomato crops (Driver et al. 2002; Rivard et al. 2012), likely due to the vertical movement of the pathogen in soil to recolonize fumigated beds (Satou et al. 2006), and fumigation is not as effective as rotation or use of tolerant cultivars. In addition, fumigation requires higher production cost for equipment and can be destructive to the environment.

Studies have examined potential alternative chemicals, focusing on the antimicrobial activities based on growth inhibition of various compounds. For instance, Feng et al. (2012) tested the growth inhibitory effect of aqueous extracts form 67 Chinese medicinal herbs against 8 genera of phytopathogenic bacteria, including *R. solanacearum*, and characterized the active components. Similarly, Vu et al. (2013) characterized gallotannin derivatives from *Sedum takesimense* and recorded their minimum inhibitory concentration (MIC) for 9 genera of phytopathogenic bacteria; Bai et al. (2016) examined the anti-microbial effects of *Syringa oblata* extract against *R. solanacearum* based on growth inhibition and total bacterial protein content. In more recent studies, in addition to simply attempting to inhibit bacterial growth, other aspect involved with pathogenicity have been used as indicators to screen for potential chemicals. Yang et al. (2016) examined the antimicrobial effect of hydroxycumarins against *R. solanacearum* and quantified the expression of important virulence genes such as *phcA* and genes involved with type III secretion system regulation. However, they did not examine such effects with any

bioassay with plants. Puigvert et al. (2019) determined the effect of several plant extracts and some molecules described as type III secretion system (T3SS) inhibitors of bacterial animal pathogens against *R. solanacearum*. They found that salicylidene acylhydrazides can inhibit T3SS through inhibition of the regulator *hrpB* and limit *R. solanacearum* multiplication *in planta*. These studies suggested potential new compounds for bacterial wilt management. However, without experimenting under field conditions and evaluating their environmental impacts, it is still a long way to go for developing novel chemical control agents.

Combining various chemical compounds for the control of bacterial wilt was also considered in some research. A series of lab and field trials to evaluate the efficacy of thymol in combination with the plant systemic acquired resistance inducer, acibenzolar-S-methyl (ASM; Actigard 50 WG) was tested in Florida tomato production systems (Pradhanang et al. 2003; Ji et al. 2005; Hong et al. 2011). Based on their study, the combination of thymol fumigation, ASM, and using moderately resistant cultivar significantly reduced disease. However, such system has not been tested on other crops.

Biological control

Biological control has been investigated extensively as a means of controlling bacterial wilt. Despite promising results in controlled conditions, similar success often does not replicate in the field (Saddler 2005). Biocontrol agents are usually examined for the potential to outcompete the pathogen through various mechanisms, such as their ability to produce certain enzymes (Elhalag et al. 2016), antibiotics, volatiles (Tahir et al. 2016), siderophores, or colonize plant roots more effectively and promote plant growth (Haas and Défago 2005; Raaijimakers and Mazzola 2012).

Most biological control agents tested between 1990 and 2000 were focused on saprophytic bacteria, including *Pseudomonas spp*, *Burkholderia spp*., *Streptomyces spp.,* and *Bacillus spp*. The reason behind using these genera was that these organisms are adapted to survive in the same plant microenvironment and have the potential to exploit conditions which favor the pathogen (Saddler 2005). However, the competitiveness of these organisms was greatly affected by environment, and the exact physiology involved in competing in certain environments is not well understood.

A more recent study tested the potential of using an unmarked Δ*phcA* mutant strain of *R. solanacearum* as biocontrol agent (Chen et al. 2015). Although it is still limited in controlled condition (potting in greenhouse and hydroponic system), when inoculating tomato plants with Δ*phcA* mutant 3-days before inoculating wild-type strain, disease incidence was reduced significantly. RT-PCR analysis indicated that when inoculated with the non-pathogenic Δ*phcA* mutant, plant genes related to salicylic acid signaling (GluA and PR-1a) were upregulated. The authors suggest that induced plant resistance by the nonpathogenic mutant is the reason for the reduced disease incidence. Inducing plant natural resistance with non-pathogenic strains has the potential to be combined with using partial resistant cultivars, but using transgenic methods to acquire such a strain and using it in the field may raise questions. While a spontaneous mutant may arise and have been tested in lab conditions, such a strain may not have the same level of fitness in the field (Quezado-Soares and Lopes 1994).

Besides bacteria, researchers have also tried to use bacteriophages to control *R. solanacearum* (Álvarez and Biosca 2017). When *R. solanacearum* cells are infected with filamentous phage ϕRSM3, the infected-cells were not able to cause wilting symptoms on tomatoes in greenhouse and enhanced the expression of pathogenesis-related genes in tomato

plants (Addy et al. 2012a). However, if bacterial cells were infected with a different filamentous phage, φRSS1, *R. solanacearum* become more virulent on host plants (Addy et al. 2012b). These data clearly indicated that the interaction between phage and *R. solanacearum* may yield diverse interactions. With the known diversity in *R. solanacearum* species complex, Murugaiyan et al. (2010) characterized the host specificity of 15 filamentous phages from soil samples on 9 R. solanacearum strains from different races and biovars. They found one phage that can infect all 9 strains of *R. solanacearum*, but this research again emphasized the potential variation of phage-bacterium interaction.

Recent studies have also examined the combining effect of biological agents such as *Bacillus amyloliquefaciens* or *Bacillus subtilis* with bio-organic fertilizers (Ding et al. 2013). Although the study only presented data from one year of field trials in two potato fields, which demonstrated exceptional control efficiency of 84.6%, the idea of combining soil amendment with biocontrol agent, which provides a better soil environment for the biocontrol agents does seem to have great potential. With the new understanding of rhizosphere microbes through genetic analysis, perhaps instead of trying to isolate single microorganisms as biocontrol agents, understanding the rhizosphere microbiome, and trying to alter soil environments in the crop rhizosphere to induce beneficial microbes would be a better approach.

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CHAPTER II. Using novel chemical compounds to enhance control of bacterial wilt caused by *Ralstonia solanacearum*

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Abstract

Bacterial wilt, caused by *Ralstonia solanacearum* (Rs), is a devastating disease of many valuable crops around the world with the only available chemical control option being the use of soil fumigants. In this study, three chemical compounds known to have anti-microbial and anti-biofilm activities were examined: 3-indolyacetonitrile (IAN), *p*-benzoquinone (pBQ), and 6,7-dihydroxycoumarin (6-7-D). The minimal inhibition concentration against Rs for each compound was determined to be 80 μM, 25 μM, and 125 μM for IAN, pBQ and 6-7-D, respectively. Sub-inhibitory concentrations, 20 μM for IAN, 12.5 μM for pBQ, and 50 μM for 6-7-D, were used to examine bacterial growth in broth culture. IAN was significantly more effective in inhibiting bacterial growth compared to pBQ and 6-7-D. There was no difference in colony morphology when Rs was grown on media amended with sub-inhibitory concentrations of each compound. Biofilm formation was enhanced when Rs was treated with IAN and 6-7-D. It was shown using fluorescence microscopy with the GFP-labeled W7 strain that IAN limited bacterial colonization of plant roots the most compared to pBQ and 6-7-D. In laboratory experiments, tobacco cultivars K326 and Speight168 were treated with the compounds 48-hours before, at the same time or 48-hours after inoculation. IAN was most effective in limiting disease incidence among the three tested compounds, and treating at the time plants were inoculated offered the most suppression regardless of the compound. In greenhouse experiments, disease progression was slower in plants treated with IAN and pBQ than the untreated or plants treated with 6-7-D in both K326 and Speight168. Our results suggest there may be potential new compounds to control bacterial wilt disease.

1. Introduction

Bacterial wilt (BW) caused by *Ralstonia solanacearum* is an important soil-borne disease of many economically important crops in tropical and subtropical regions of the world. The pathogen has a host range of more than 50 families (Kelman 1953; Hayward 1994) and has considerable genetic diversity worldwide (Fegan and Prior 2005). In the Southeastern United States, the disease is a limiting factor for important solanaceous crops including tomato, tobacco, and eggplant (Rivard et al. 2012). To reduce the yield losses due to BW, an integrated disease management program is required (Saddler 2005). Such a program includes the use of pathogen-free planting material, planting resistant cultivars, rotating susceptible crops with non-host crops, and soil fumigants (Fortnum and Martin 1998; Yuliar and Toyota 2015). However, the wide host range of *R. solanacearum* limits rotational options. In severely infested fields, an effective rotation could require multiple years not planted with high value host crops (Kelman, 1998). The use of resistant cultivars is one of the most cost-effective strategies, but resistance to BW in tomato and tobacco are quantitative and strongly affected by environmental conditions such as soil temperature, pH, and moisture (Scott et al. 2005; Bittner et al. 2016). Although soil fumigants provide good control in a given year, they have been reported to have limited success because of vertical movement of *R. solanacearum* in soil that may allow the bacterium to recolonize fumigated beds quickly (Driver and Louws 2002; Satou et al. 2006). Furthermore, use of fumigants is in decline due to restrictions imposed by the Environmental Protection Agency and other international regulatory organizations.

Research on other means of control of BW is underway. Due to the concerns of modern society about application of toxic treatments that could potentially contaminate the soil and crops, methods with potential low environmental impact are sought (Yuliar et al., 2015; Bai et

al., 2016). For instance, antimicrobial plant essential oils have been suggested as eco-friendly agents to decrease the tomato bacterial wilt (Ben-Jabeur et al. 2015; Pradhanang et al. 2003; Vu et al. 2013). Acibenzolar-S-methyl (ASM) has been proposed to induce systemic resistance (Hacisalihoglu et al. 2007). The combination of ASM and thymol has been reported to significantly reduce the incidence of disease and increase the yield of tomato crops (Hong et al. 2011). More recently, novel materials that suppress bacterial wilt have been investigated such as silicon, cold plasma, vitamins, sulfone and coumarin derivatives (Wang et al. 2013; Denslow et al. 2005; Yang et al. 2016). These novel materials fall usually into one of two mechanisms of action, that is induce plant resistance or disrupt important bacterial functions.

One important function of *Ralstonia solanacearum* is biofilm formation. It occurs when microcolonies aggregate and surround themselves by an extracellular matrix (Mori et al. 2015; LaSarre and Federle 2013). A mutant strain with decreased ability to colonize intercellular space and form a biofilm led to loss of virulence on tomato plants (Mori et al. 2015). In addition, a mutant lacking extracellular nucleases, which formed abnormally thick biofilms in the xylem, was also reduced in virulence on tomato plants and did not spread in tomato stems as well as the wild-type strain (Tran et al. 2016). Thus, disrupting biofilm formation appears to be a potential mechanism for controlling bacterial wilt. Disruption of biofilm development by a wide variety of natural and synthetic compounds has been studied predominately on human pathogenic species of *Burkholderia* and *Pseudomonas* (Lesic et al. 2007; Rudrappa and Bais 2008; Tateda et al. 2001). Brackman et al. (2009) demonstrated that 6,7-dihydroxycoumarin (6-7-D) and p-Benzoquinone (pBQ) were effective in inhibiting biofilm formation in the common environmental bacteria *Escherichia coli*, *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. 3-indolylacetonitrile (IAN) inhibited biofilm formation of *Xanthomonas citri* subsp.

citri on citrus leaves (Li and Wang 2014). Recently Yang et al. (2016) demonstrated that hydroxycoumarins had activity against *R. solanacearum* and suggested that reduction in biofilm formation is the most likely mechanism of action.

The present study was carried out to evaluate and compare *in vitro* and *in vivo* activity of 3-indolylacetonitrile (IAN), 6,7-dihydroxycoumarin (6-7-D), and p-Benzoquinone (pBQ) against *R. solanacearum*. Specifically, we examined the effect of the compounds on *i)* growth and colony morphology of *R. solanacearum; ii)* root colonization, by use of a GFP-labeled strain of *R. solanacearum*; and *iii*) level of bacterial wilt incidence in tobacco plants treated with these compounds in laboratory and greenhouse experiments. The compounds were chosen based on the fact that their antibacterial activity is attributed to biofilm inhibition, although the exact mechanism of action was not investigated in the research presented here.

2. Material and Methods

2.1 *Ralstonia solanacearum* strains and growth conditions. *Ralstonia solanacearum* strain W7, previously characterized as phylotype IIA sequevar 7 (Tseng and Mila 2012), was isolated on 2,3,5-triphenyltetrazolium chloride (TZC) medium (Kelman 1953) from a diseased tomato stem collected in North Carolina in 2011 and stored in 15% glycerol at -80ºC. For routine culturing and preparation of inoculum, the bacterium was streaked on BG agar medium (1% Bacto peptone, 0.1% casamino acid, 0.1% yeast extract, 0.5% glucose, and 1.6% agar) and then incubated at 28 ºC in dark immediately after removing from the -80ºC glycerol stock.

2.2 Preparation of compound stock solutions. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) was used to prepare stock solutions of 3-indolylacetonitrile (IAN), 6,7-dihydroxycoumarin (6-7-D), and p-Benzoquinone (pBQ) (Sigma-Aldrich, St. Louis, MO). Stock

solutions at 64mM for IAN, and 10mM for 6-7-D and pBQ were filter sterilized and stored at -20 ^{o}C.

2.3 Determination of Minimal Inhibition Concentrations (MIC). *Ralstonia solanacearum* was grown on BG agar medium at 28°C for 48 h. Bacterial cells were scraped from the surface of the BG agar medium and standardized to an optical density at 600 nm (OD_{600}) of 0.1 (approximately equal to 10^8 CFU/ml) in BG broth. A volume of 100 µl of the standardized suspension was aliquoted into 6 ml BG broth in test tubes, amended with the stock solutions of each compound diluted in a series of 2-fold dilutions. The tubes were incubated at 28°C for 48 h and absorbance at 600 nm was measured. To examine if the compounds affect cell viability, sub-samples of 10 µl, taken at the beginning and end of the incubation, were spotted on TZC agar medium, incubated for 48 h, and examined for bacterial growth. The lowest concentration that resulted in complete growth inhibition based on OD_{600} values after 48 h of incubation was defined as the MIC for each compound. For all further experiments, a single sub-MIC concentration was selected for each compound. Sub-MICs were determined as the highest concentration that allowed R. *solanacearum* to grow to a cell density of OD_{600} >1.0 within 48 h. The experiments were done with at least three biological replicates.

2.4 Evaluation of compounds on *R. solanacearum* growth. The effect of the compounds on *R. solanacearum* growth at sub-MIC was investigated in 50 ml BG broth cultures. *R. solanacearum* was grown on BG agar medium at 28°C for 48 h, washed from the plates, and adjusted to an OD_{600} of 0.1 using fresh BG broth. A volume of 100 µl of the standardized suspension was aliquoted into 50 ml BG broth in 125 ml flasks, amended with the compounds at sub-MIC, and incubated at 28°C with 200 rpm agitation. Samples of 100 µl were taken from each flask every 4 h in the first 12 h and every 2 h thereafter for serial dilution and spread plating on

TZC agar medium. Single colonies were counted after incubating at 28°C for 36 h. The experiment was repeated twice, with three replications per compound and concentration for each experiment. For each time point, the counts of three replications were averaged and back-transformed to colony forming units per ml (CFU/ml) based on the dilution rate. The logarithm of the relative population size, $y = \ln (N/N_0)$, where N is the population size at a given time point and N_0 is the initial population size, was calculated from CFU/ml.

2.5 Effect of compounds on colony morphology. *Ralstonia solanacearum* colonies were grown on BG agar medium amended with the compounds at sub-MICs and examined for twitching motility. Petri dishes without lids were placed on the stage of an upright light microscope (Olympus BX41). Digital images were acquired with a Q-color 3 camera system (Olympus® America, INC).

2.6 Biofilm assay. Biofilm formation of *R. solanacearum* when treated with the compounds was performed in 96-well PVC microtiter plates as previously reported (Yao and Allen, 2007). Briefly, 5 µl of 0.1 OD_{600} *R. solanacearum* suspension were seeded into each well of the plate containing 95 µl of fresh BG amended with the compounds at sub-MIC. The plates were incubated at 28°C without agitation for 24 h. Bacterial growth were recorded as absorbance at 600 nm (OD_{600}). Biofilms were then stained with 1% w/v crystal violet, dissolved in 95% ethanol and quantified by absorbance at 590 nm (OD_{590}). The assays were repeated to include at least three biological replicates.

2.7 In planta evaluation. *Microplates.* Two flue-cured tobacco cultivars with different levels of resistance to *R. solanacearum* were used: K326 (low resistance) and Speight168 (high resistance) (Mila and Radcliff 2015). Tobacco plants were grown in 12-well tissue culture (TC) plates (Corning Incorporated, Corning, NY) using the method described by Katawczik and Mila

(2012). Five to six seeds were placed in each well with 3.5 cm^3 of perlite and 1.5 ml of sterilized deionized water (SDW). To maintain high relative humidity, SDW was added into the space between each well and TC plates were covered with clear plastic wrap. Plants were placed at room temperature (20 to 25 ºC) with a 12 -h light cycle. A 0.5 ml solution of 200-ppm N fertilizer (Bulldog water-soluble fertilizer 20-10-20; Chilean Nitrate Corporation, Northfolk, VA) was added to the wells of each plate 10 days after seeding and once a week thereafter. Water was added to wells once a week, or as needed.

Inoculum was prepared by growing *R. solanacearum* on BG agar medium at 28°C for 48 h. Cells were washed from the plates and adjusted to approximately 10^8 CFU/ml with SDW. Roots of 25-day-old seedlings were injured before inoculation by stabbing a sterilized scalpel around each seedling. Immediately after the roots were injured, 0.5 ml of the bacterial suspension was added to each well of the plate.

IAN at 80 µM, pBQ at 25 µM, and 6-7-D at 125 µM were individually prepared by diluting the stock solutions in 10 ml of SDW. Each well was treated with 0.5 ml of the compound dilution, and treatments were applied at three different time points: 48 h before inoculation, at the time of inoculation, and 48 h after inoculation. Non-inoculated plates were used as the negative control and plates with the compounds but without *R. solanacerum* were used to examine possible phytotoxic effects caused to the plants by the compounds.

Numbers of plants with bacterial wilt symptoms such as wilting, leaf chlorosis, and necrosis on the root were observed and recorded 10 and 20 days after inoculation for the microplates. *Greenhouse*. Tobacco cultivars K326 and Speight168 were used. Tobacco plants were grown from seed directly in 3.5-by-3.5-cm cell flats filled with a mixture of soil, sand and Fafard 4M potting mix (Fafard Inc., Agawam, MA) (1:1:1) and fertilized 3 and 5 weeks after seeding. Each

plant was supplied with 200 ppm water soluble fertilizer (20-10-20, N-P-K). Inoculation was conducted when tobacco plants were 6 weeks old with the method reported above. Five ml of *R. solanacearum* suspension was used per plant. There were three replications of six plants per treatment.

Plants were treated with IAN at 31.25 µg, pBQ at 6.75 µg, or 6-7-D at 55.67 µg, respectively, at the same time that plants were inoculated, which is the 5× rate compared to the rate used in the microplates experiments. In preliminary greenhouse experiments, we used 1× and 5× of the rates used in the microplates experiments. At the 1× rate, none of the compounds provided significant BW control compared to the untreated inoculated control, whereas the 5x rate did (data not shown). Non-inoculated plants were used as the negative control and inoculated non-treated as positive controls. Twelve plants were analyzed at each time point, numbers of plants with bacterial wilt symptoms were observed and recorded 7, 10, 12, 14, and 17 days after inoculation. The experiment was repeated twice.

Disease incidence data were logit transformed and analyzed using the GLM procedure of SAS (SAS version 9.4; SAS Institute, Cary, NC). Bartlett's test was used to confirm homogeneity of variance between experiments. Fisher's least significant difference test (P = 0.05) was used to test differences between treatments.

2.8 Creation of *R. solanacearum* W7-gfp7. For the histological studies, *R. solanacearum* W7-gfp7, which expresses green fluorescent protein (GFP-mut2) from a constitutive pathogen promoter, was used. *R. solanacearum* W7-gfp7 was created by natural transformation with genomic DNA from AW1-gfp38 (Liu et al. 2001). Total genomic DNA extraction was conducted with the Qiagen DNeasy blood and tissue kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. The pathogenicity of the GFP-labeled strain, W7-

gfp, was confirmed using the *in planta* inoculation method described above (Katawsczik and Mila 2012). Briefly, fifteen 25-day-old seedlings of susceptible cultivar K326 were inoculated with 0.5 ml of 10^8 CFU/ml suspension of strain W7-gfp immediately after the roots were injured. Tobacco seedlings inoculated with wild-type W7 isolate were used as a negative control. Wilting of the leaves and necrotic roots was observed 10 days after inoculating with W7-gfp and wild-type W7, which confirmed a pathogenicity of W7-gfp comparable to wild-type W7.

2.9 Histological studies with *R. solanacearum* W7-gfp7. To understand if the compounds interfere with *R. solanacearum* ability to infect tobacco roots, the GFP-labeled strain W7-gfp3 was used to inoculate tobacco cultivar K326 as described in the *in planta* evaluation section. Plants were treated with IAN at 80 µM, pBQ at 25µM, or 6-7-D at 125µM, respectively, at the same time that plants were inoculated. Seedlings were examined 12, 24, and 48 h after inoculation because under favorable environmental conditions, *R. solanacearum* can invade roots and spread to the hypocotyl and lowest petioles of tomato and tobacco plants within 2 days (McGarvey et al. 1999, Bittner et al. 2016). Twelve tobacco seedlings were arbitrarily selected and examined on each observation time. Seedlings were washed in SDW to remove excess debris, perlite, and seed coats from the root system. The leaves from each seedling were removed, leaving the epicotyl, hypocotyl, and the whole root system intact. Each plant was placed on a glass slide and the roots observed for fluorescence with a microscope equipped with a GFP filter (Nikon Eclipse T*i*-E; Nikon Instruments Inc., Tokyo). The number of plants treated with each compound with roots infected by *R. solanacearum* was recorded. The study was repeated twice.

3. Results

3.1 Minimal inhibition concentrations (MIC). Among the three compounds tested, pBQ had the lowest MIC equal to 25 μM, followed by IAN and 6-7-D with MIC of 80 μM and 125 μM, respectively. Cells treated with 50 μM and 100 μM of pBQ were not viable after 48 h. With 6-7-D, cell viability decreased as concentration of 6-7-D increased above the MIC value. Cells amended with 250 μM of 6-7-D in broth culture were viable after 48 h, but not with 500 μM. In the case of IAN, cells were still viable after incubation in 160 μM, a two-fold MIC value.

3.2 Effect of sub-MIC compound concentrations on *R. solanacearum* growth. In untreated BG broth, *R. solanacearum* reached the maximum population density within 16 h and decreased. When cultures were amended with pBQ at 12.5 μM or 6-7-D at 50 μM, the growth curve exhibited a trend similar to the one observed in the untreated control (Figure 1); however, the population size reached the maximum at 18 h instead of 16 h. After that time point population size dropped rapidly (Figure 1). IAN at 20 μM inhibited bacterial growth. During the 24 h period of measurement, the population sizes of bacterial cultures in IAN-amended medium were smaller than the untreated control (Figure 1).

3.3 Effect of compounds on colony morphology. Colonies of *R. solanacearum* had distinct pigmentation in the center of the colonies and multiple irregular projections when examined at 100 X magnification after 36 h of incubation at 28ºC on BG agar medium (Figure 2A). When bacteria were grown for 36 h on BG agar medium amended with 12.5 μM of pBQ or 50 μM of 6-7-D no differences in colony morphology were observed compared to the untreated control (Figure 2B-C). Bacterial colony growth on BG agar medium with IAN amended at 20 μM was greatly inhibited. After 60 h of incubation, colonies were still about pin-head size, with less pigmentation in the center (Figure 2D), an indication of growth inhibition.

3.4 Effect of the compounds on biofilm formation. After *R. solanacearum* cells were incubated in BG broth at 28 ºC without agitation for 24 h, biofilm rings formed at the air-liquid interface. Interestingly, when bacterial cells were grown in the presence of 20 µM IAN, *R. solanacearum* formed significantly more biofilm than untreated cells (Figure 3). When bacterial cells were grown with BG amended with 50 µM 6-7-D, biofilm production was also significantly increased comparing to untreated cells. 12.5 µM of pBQ did not significantly affect *R. solanacearum* biofilm formation on PVC surfaces.

3.5 Effect of the compounds on BW incidence. *Microplates.* Bacterial wilt symptoms were first observed 5 to 7 days after inoculation. IAN effectively suppressed BW development 20 days after inoculation in both cultivar K326 with low resistance and cultivar Speight 168 with high resistance (Table 2). Treating the plants with IAN at 80 µM before, at the same time, or after inoculation all significantly reduced disease incidence compared to the untreated control ($P<0.0001$), regardless of the cultivar inoculated. Among the three treatments, plants treated with IAN before, or at the time of inoculation resulted in a significantly ($P<0.05$) lower disease incidence compared to the incidence recorded in plants treated 48 h after inoculation.

In the case of pBQ, disease incidence was 50% less in Speight168 compared to the untreated control 10 days after inoculation when treated at the time of inoculation. The effect of applying pBQ at the time of inoculation on reducing disease incidence was still significant 20 days after inoculation ($P<0.00001$), but none of the other two treatments were effective (Table 3). On cultivar K326, applying pBQ before, at the same time, or after inoculation was effective in reducing disease incidence 10 days after inoculation. However, except for the co-inoculation treatment, where disease incidence was 52% less compared to the untreated control, the effect of pBQ did not persist 20 days after inoculation (Table 3). When treated with 6-7-D (at 125µM),

disease incidence on cultivar K326 was on a range between 58.3% and 65% even 20 days after inoculation, but on Speight168 no treatment significantly reduced the disease compared to the untreated control (Table 4). Overall, regardless of the cultivar inoculated or compound used, when a compound was applied at the time of the inoculation this was always the treatment suppressing the disease the most, although it was not always statistically different than applying the compounds before or after inoculation. Further, the effect of the compounds was greater when coupled with BW resistance except for the case of 6-7-D. No phytotoxicity was observed in any plants of the two cultivars.

Greenhouse. On cultivar K326, IAN treatment significantly reduced disease incidence 7, 10, and 12 days after inoculation ($P<0.05$) but was not significantly different from the untreated control at 14 and 17 days. A similar pattern was observed when treated with pBQ. However, similar differences in disease incidence were not observed when IAN or pBQ was used on cultivar Speight 168. On both cultivar K326 and Speight 168, 6-7-D did not have any significant effect on disease incidence during the 17-day period of the experiments when compared with the untreated control (Figure 4A & B).

3.6 Histological studies with *R. solanacearum* W7-gfp7. On the non-treated control, 41.6% of the tobacco seedlings examined had roots colonized by *R. solanacearum* 12 hours after inoculation. When treated with pBQ at 25 μM or 6-7-D at 125 μM, there was no significant difference in the number of plants colonized by the bacteria compared to non-treated control. However, when treated with IAN at 80 μM, only 8.8% of the examined tobacco seedlings were colonized by *R. solanacearum* (Table 5). When examining the plants 24 and 48 h after inoculation, number of plants with roots colonized by the bacteria increased to 75% and 83%, respectively in the untreated control. At 48 hours, colonization of the bacteria on multiple

epidermal root cells could be observed on untreated tobacco roots (Figure 5A & B). No significant differences in the number of plants with roots colonized by the bacteria were observed in plants treated with 6-7-D or pBQ (Table 5; Figure 5E-H). At 48 h, the number of plants with bacterial colonization when treated with pBQ at 25µM or 6-7-D at 125µM was 83% and 70.67%, respectively. The effect of IAN on inhibiting bacterial colonization on plant roots was significant even 48 hours after inoculation. At 48 hours, number of plants with roots colonized by the bacteria when treated with IAN at 80 µM was 20.8% less (P=0.05) than the untreated control or the pBQ and 6-7-D treatments (Table 5; Figure 5C & D).

4. Discussion

In this study, we evaluated and compared in vitro activity of 3-indolylacetonitrile (IAN), 6,7-dihydroxycoumarin (6-7-D), and p-Benzoquinone (pBQ) against the bacterial wilt pathogen, *R. solanacearum*. Among the three compounds investigated in this study, IAN was the most effective against *R. solanacearum* in inhibiting bacterial growth, colonization of the roots, and reducing disease incidence.

Previous studies have demonstrated that IAN repressed expression of chemotaxis and motility-related genes in *Xanthomonas citri* subsp. *citri* (Li and Wang 2014) and in *Pseudomonas aeruginosa*, an opportunistic human bacterial pathogen (Lee et al. 2011). Chemotaxis is an important trait for virulence and pathogenicity in *R. solanacearum* (Yao and Allen 2006). A non-flagellated mutant strain of *R. solanacearum* caused less disease when used to inoculate tomato plants through soil drench (Tans-Kersten et al. 2001). Our fluorescence microscopy results indicate that IAN greatly reduced *R. solanacearum* colonization on tobacco roots. Inhibited chemotaxis and motility due to IAN application could be a plausible explanation

for the reduced root colonization. Future experiments to investigate gene expression are needed to conclude the effect of IAN.

In addition to affecting initial plant infection, treating plants with 80 μM IAN 48 h after inoculation was still sufficient to significantly reduce disease incidence ($P<0.0001$). Under favorable environmental conditions, *R. solanacearum* can invade roots and spread to the hypocotyl and lowest petioles of tomato and tobacco plants within 2 days (McGarvey et al. 1999; Bittner et al. 2016). Normanly et al. (1997) demonstrated that on *Arabidopsis thaliana*, IAN has auxin-like effects, and the exogenous IAN can be converted to IAA *in vivo* in aseptically-grown Arabidopsis plants. High levels of IAA were found in culture media and in tobacco plants infected with *R. solanacearum* (Sequeira and Kelman, 1962; Sequeira and Williams 1964). Auxin has been shown to be involved in plant defense response signaling and represses the expression of some pathogenesis-related (PR) genes (Jouanneau et al. 1991; Kazan and Manners 2009). It is likely that IAN can be absorbed through the roots of treated tobacco plants, but the progression and fate of this exogenous IAN in the plant is not clear. Previous research has demonstrated that IAN will not be metabolized into indo-3-acetic acid (IAA) in tobacco due to the lack of a specific nitrilase gene (Schmidt et al. 1996).

The stability of IAN treated to plants is also unclear. When IAN was amended to pure cultures of *E. coli* or *P. aeruginosa*, the concentration remained stable in the culture for the duration of the experiment (Lee et al. 2011). Our results demonstrated that treating plants with IAN 48 h before inoculation reduced disease incidence significantly ($P < 0.05$). This indicates that the compound may have a lasting effect after treating in non-sterile environments. However, when treated plants were grown under greenhouse conditions, IAN was only able to reduce disease incidence for the first 12 days after inoculation. A multitude of factors may have

contributed to these differences. Chemical compounds may directly interact with soil through adsorption and covalent bond formation (Bollag et al. 1992), which greatly affects its availability to a crop. The plants used in the greenhouse experiments were much larger in size comparing to the plants grown in microplates, therefore they may require higher quantity of the active ingredient or multiple applications over time. Although further research is required to determine the environmental fate and the most effective approach to applying IAN, it is encouraging to discover that IAN delays disease development. To our knowledge, this is the first reported attempt to apply IAN in the soil environment for disease control purposes.

The other two compounds, pBQ and 6-7-D, were less effective in reducing disease incidence than IAN. 6-7-D, also known as esculetin, is a derivative of coumarin. Coumarins are a well-known family of compounds with structural variety and a wide range of activities (Savola 2012). Yang et al. (2016) demonstrated that 6-7-D inhibits the growth and expression of some pathogenicity-associated genes of *R. solanacearum*. However, the study focused on the effects of 6-7-D on the pathogen and did not test the effect of the compound in reducing disease incidence. Yang et al. (2016) reported the MIC for 6-7-D on a *R. solanacearum* strain phylotype I, race 1, biovar 3 to be 192 mg/L, which was higher than the MIC value found in our study. The difference in MIC could indicate that different phylotypes of *R. solanacearum* have varied reaction to 6-7-D.

Our hypothesis in the current study was that use of these compounds with a resistant cultivar would further reduce BW incidence. Indeed, in our microplate in planta assay, treating the resistant cultivar Speight168 with IAN or pBQ enhanced the suppression of disease compared to cultivar K 326 or the untreated control whereas no effect was observed with 6-7-D. However, similar results were not observed under greenhouse conditions. The compounds did

not further suppress disease incidence in cultivar Speight 168. This suggests that different cultivars may interact differently with the compounds, particularly as the plants become more mature.

Managing BW has always been difficult in fields where the pathogen is endemic. On tobacco, the use of soil fumigants and resistant cultivars are recommended for effective control of the disease (Mila and Radcliff 2015). In agricultural systems, an integrated pest management strategy is crucial (Yuliar and Toyota, 2015). Whether these compounds, alone or in combination with cultural practices, will ever be useful as effective management strategies remains to be determined in future field studies.

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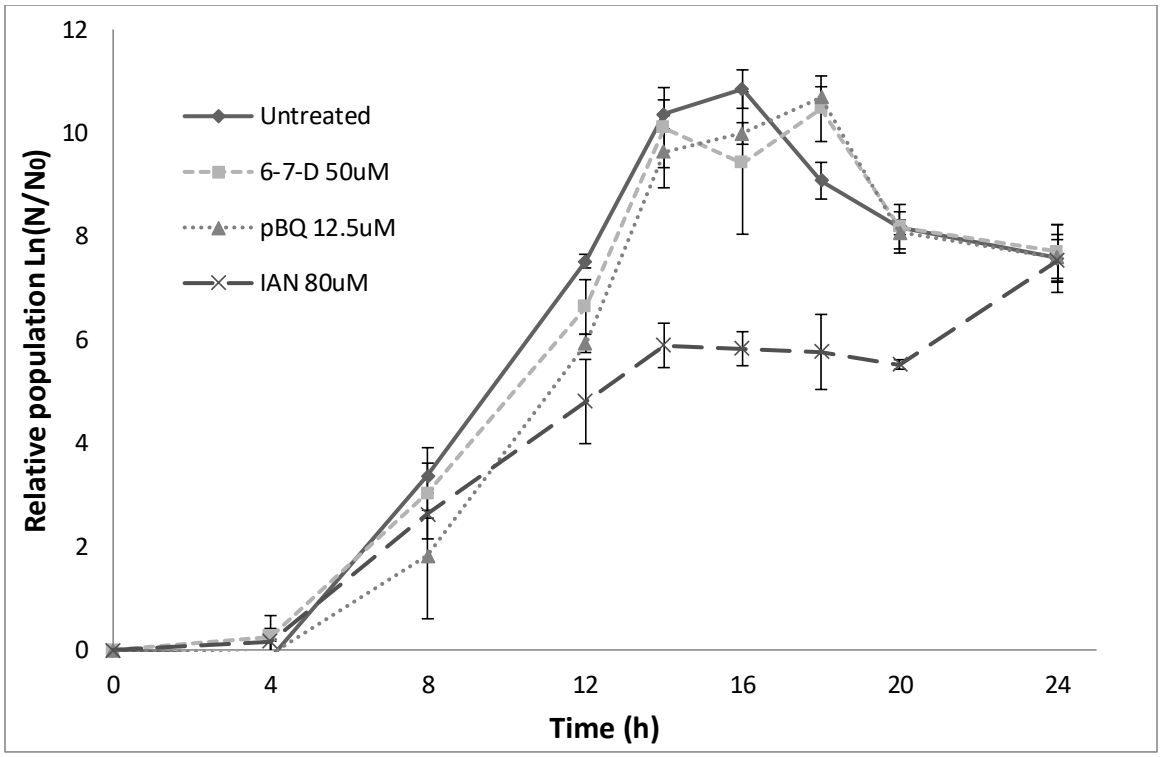


Figure 1. Growth curve analysis for *R. solanacearum*. The bacteria were grown in the presence of 50 μM of 6-7-D, 12.5 μM of pBQ, or 20 μM of IAN, respectively. The y-axis is the logarithm of the relative population size, ln (N/N_0), where N is the population size at a given time point and N_0 is the initial population size. The x-axis is the time in hours that bacteria were exposed to each compound. Error bars represent the standard error. The experiment was repeated twice.

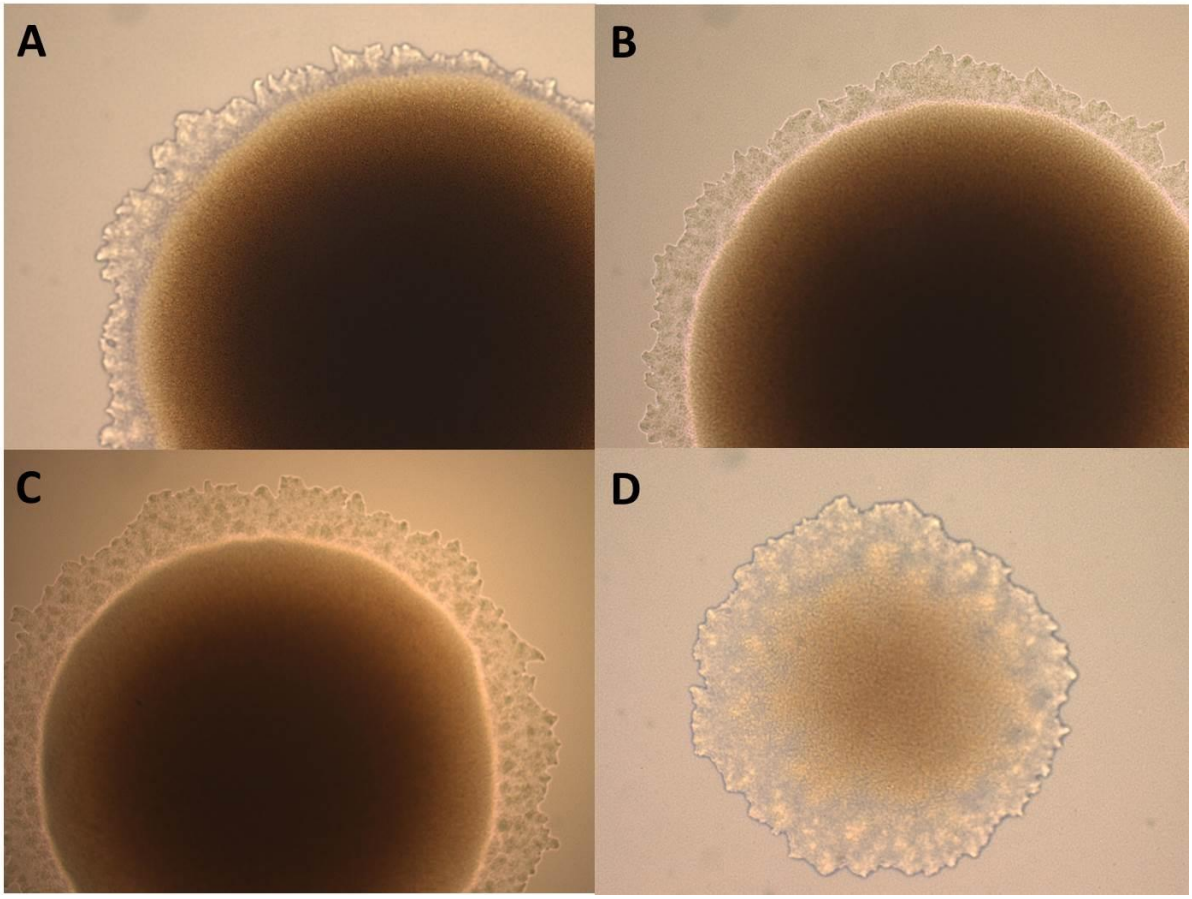


Figure 2. Colony morphology of *R. solanacearum* strain W7 incubated for 36 h on BG agar media non-amended (A), amended with 12.5 µM of p-Benzoquinone (B), amended with 50 µM of 6,7-dihydroxycoumarin (C) and after 60 h of incubation, amended with 20 µM of 3-indolylacetonitrile (D).

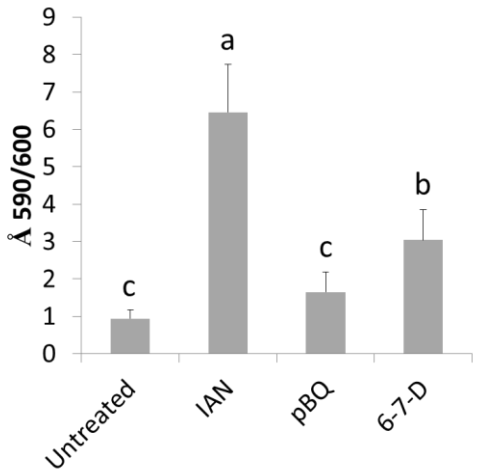


Figure 3. *R. solanacearum* biofilm production was increased when treated with 20 µM of 3-indolylacetonitrile (IAN) or 50 µM of 6,7-dihydroxycoumarin (6-7-D) on PVC surfaces, while 12.5 µM of p-Benzoquinone (pBQ) had no significant effect. Biofilm formation was quantified by measuring A_{590} of crystal violet-stained wells rinsed with ethanol normalized to cell density (A_{600}). Each column is the mean of three individual experiments with two replicates per treatment. The error bars represent the standard error of the mean. Columns with different letters are significantly different according to the Fisher lease significant difference test ($P < 0.05$).

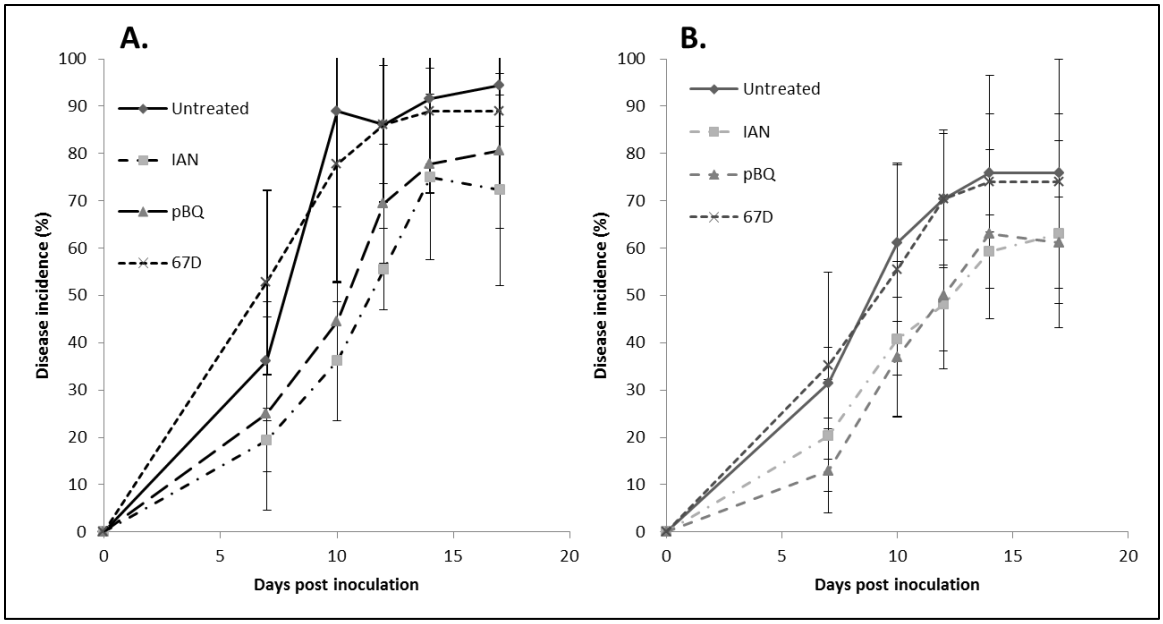
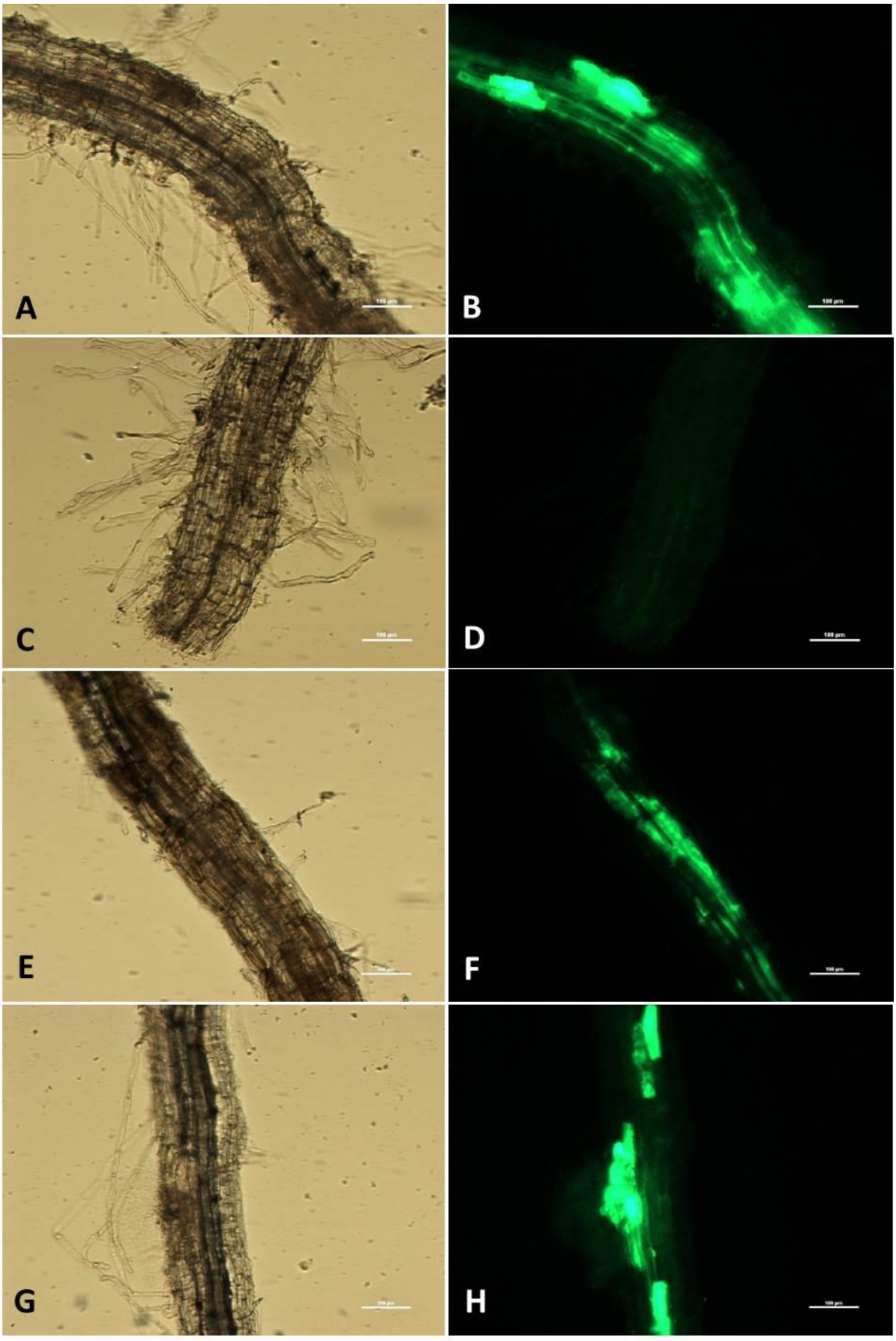


Figure 4. Bacterial wilt incidence for greenhouse trials with tobacco cultivar K326 (A) and Speight 168 (B). Plants were treated with 31.25 µg of 3-indolyacetonitrile (IAN), 6.75 µg of *p*-benzoquinone (pBQ), or 55.67 µg of 6,7-dihydroxycoumarin (6-7-D). Mean and standard error of three experiments are shown.

Figure 5. Light and fluorescence microscopy of tobacco roots colonization with the GFP-labeled strain W7-gfp3 of *Ralstonia solanacearum*, 48 h post-inoculation. Areas with green florescence indicate sites of bacteria colonization. (A, B) Untreated tobacco root with multiple epidermal cells colonized by the bacteria. (C, D) Plant treated with IAN at 80 μM with no sign of *R. solanacearum* colonization at wounded site where the root was cut off. (E, F) Plant treated with 6-7-D at 125 μM and (G, H) plant treated with pBQ at 25 μM. Both 6-7-D and pBQ had similar bacterial colonization as untreated plants. Bar = 100μm.


A
B
C
D
E
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H

Table 1. Effect of application time of 3-indolylacetonitrile (IAN) at 80 μM on the percent incidence of bacterial wilt on tobacco caused by wild-type strain W7 of *Ralstonia solanacearum* 10 and 20 days after inoculation under laboratory conditions [z]

Application time	**10 days**		**20 days**	
	K326	**Speight 168**	**K326**	**Speight 168**
Untreated	46.67±10.33a	20.0±8.94a	83.33±27.33a	50.0±6.32a
48h before inoculation	6.67±8.16b	5.00±5.48bc	18.33±17.72c	11.67±9.83bc
At inoculation	1.67±4.08c	1.67±4.08c	21.67±4.08bc	3.33±5.16c
48h after inoculation	18.33±11.69bc	10.0±6.32b	31.67±7.53b	18.33±7.53b

[z] Bacterial Wilt (BW) incidence was recorded 10 and 20 days after inoculation for K326 with low resistance and Speight 168 with high resistance to disease. BW incidence was calculated as the percentage of plants that were completely wilted. Data shown are means of three experiments. There were two replications in each experiment. Ten plants were evaluated at each time point by replicates. Means within the same column followed by the same letter are not significantly different according to Fisher's least significant difference test ($P = 0.05$).

Table 2. Effect of application time of p-Benzoquinone (pBQ) at 25 µM on the percent incidence of bacterial wilt on tobacco caused by wild-type strain W7 of *Ralstonia solanacearum* 10 and 20 days after inoculation under laboratory conditions [z]

Time of treatment	**10 days**		**20 days**	
application	**K326**	**Speight 168**	**K326**	**Speight 168**
Untreated	45.0±5.48a	26.67±5.16a	88.33±4.08a	50.0±6.32b
48h before inoculation	26.67±5.16b	21.67±17.22ab	86.67±12.11a	66.67±13.66a
At inoculation	16.67±5.16c	13.33±8.16b	46.67±8.16b	36.67±8.16c
48h after inoculation	21.67±7.53bc	21.67±7.53ab	81.67±7.53a	55.0±5.48b

[z] Bacterial Wilt (BW) incidence was recorded 10 days and 20 days after inoculation for K326 with low resistance and Speight 168 with high resistance to disease. BW was calculated as the percentage of plants that were completely wilted. Data shown are means of three experiments. There were two replications in each experiment. Ten plants were evaluated at each time point by replicates. There were two replications in each experiment. Ten plants were evaluated at each time point by replicates. Means within the same column followed by the same letter are not significantly different according to Fisher's least significant difference test (P = 0.05).

Table 3. Effect of application time of 6,7-dihydroxycoumarin (6-7-D) at 125 µM on the percent incidence of bacterial wilt on tobacco caused by wild-type strain W7 of *Ralstonia solanacearum* 10 and 20 days after inoculation under laboratory conditions [z]

Time of treatment	**10 days**		**20 days**	
application	**K326[1]**	**Speight 168[2]**	**K326**	**Speight 168**
Untreated	51.67±16.02a	25.0±8.37a	86.67±10.33a	63.33±16.33a
48hr before inoculation	21.67±9.83b	30.0±12.65a	61.67±16.02b	70.0±16.73a
Co-inoculation	33.33±10.33b	21.67±11.69a	58.33±7.53b	63.33±15.06a
48hr after inoculation	28.33±7.53b	25.0±5.48a	65.0±8.37b	63.33±8.16a

[z] Bacterial Wilt (BW) incidence was recorded 10 days and 20 days after inoculation. BW was calculated as the percentage of plants that were completely wilted. Data shown are means of three experiments. There were two replications in each experiment. Ten plants were evaluated at each time point by replicates. Means within the same column followed by the same letter are not significantly different according to Fisher's least significant difference test (P = 0.05).

Table 4. Colonization of tobacco roots of cultivar K326 by the GFP-labeled strain W7-gfp3 of *Ralstonia solanacearum* as affected by 3-indolylacetonitrile (IAN), p-Benzoquinone (pBQ), or 6,7-dihydroxycoumarin (6-7-D), at 12, 24, and 48 h after inoculation

	Time		
Compound[z] , concentration	12 h	24 h	48 h
None	41.65±11.8 a	75.0±0 a	83.3±0 a
IAN, 80µM	8.8±11.73 b	12.3±6.08 b	20.8±5.94 b
pBQ, 25µM	29.1±17.67 a	54.15±5.87 a	83.3±11.74 a
6-7-D, 125µM	29.15±5.86 a	58.3±23.62 a	70.67±17.89 a

[z] Compounds at the given concentration were applied at the time of inoculation. Numbers represent the mean percentage of plants treated with each compound with roots colonized by *Ralstonia solanacearum* strain W7-gfp3 at 12, 24 and 48 h after inoculation. Means within the same column followed by the same letter are not significantly different according to Fisher's least significant difference test (P=0.05).

CHAPTER III. Effects of selected compounds on *Ralstonia solanacearum*, the causal agent of bacterial wilt of tobacco

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Abstract

Bacterial wilt, caused by *Ralstonia solanacearum*, is a devastating disease of many valuable crops around the world with the only available chemical control option being the use of soil fumigants. In this study, the effects of three antimicrobial compounds were examined against for their ability to interfere with expression of virulence factors and metabolic capacity of *R. solanacearum*. The compounds were 3-indolyacetonitrile (IAN), p-benzoquinone (pBQ), and 6,7dihydroxycoumarin (6-7-D). At a concentration without affecting bacterial growth, IAN was the most effective compound in limiting bacterial flagellar motility, metabolism of various carbon sources, and disease severity, yet increased the production of extracellular polysaccharide (EPS) in culture. Quantitative real-time PCR revealed that expression of crucial EPS regulators, PhcA and XpsR, concurred with the biochemical assay results. To lesser degree, 6-7-D also inhibited flagellar motility and increased EPS production in culture but did not inhibit the ability of *R. solanacearum* to metabolize different carbon sources. pBQ was least effective in interfering with virulence factors and metabolism. These results provided insight on how chemical compounds may be affecting multiple aspects of important processes in infection and disease development. Utilizing the knowledge of bacterial physiology and pathogenicity can give researchers valuable criteria for evaluating potential chemical agents, instead of simply looking for antimicrobial compounds based on growth inhibition.

1. Introduction

Ralstonia solanacearum is a soil-borne bacterial pathogen that causes lethal wilt symptoms in over 200 plant species around the world, including tomato, tobacco, potato, and native plants in warm and moist climates (Hayward 1994). Few effective chemical management options are available against bacterial wilt caused by the pathogen. In recent years, researchers have attempted to block the ability of bacteria to infect the host by inhibiting bacterial virulence factors (Rasko and Sperandio 2010). Unlike antibiotics, virulence inhibitors do not kill the pathogen and should impose less selection pressure, avoiding the rapid appearance of resistance. To evaluate the application potential of virulence inhibitors, having a comprehensive understanding of the pathogen is extremely beneficial.

Ralstonia solanacearum has been a model system for studying host-pathogen interaction for over 60 years (Genin and Denny 2012). The understanding of its important virulence factors and regulation can provide insight for evaluating novel chemical compounds. The most important virulence factor of *R. solanacearum* is the high molecular mass extracellular polysaccharide (EPS). EPS is required for *R. solanacearum* to cause wilting and to kill host plants (Denny and Baek 1991; Saile et al 1997). Recent research has demonstrated that *R. solanacearum* develops biofilm in host xylem and intercellular spaces (Mori et al. 2016; Tran et al. 2016). EPS is also associated with development of biofilm as the main part of the biofilm matrix. It provides mechanical stability of biofilm, and assists bacterial cells attachment to surfaces (Flemming and Wingender 2010) and the fine-tuning of EPS and other components of the biofilm matrix is also important for *R. solanacearum* dispersal in the xylem vessels (Tran et al. 2016). High level of EPS expression involves multiple regulatory pathways of the bacteria. Among those, the quorum-sensing response regulator, PhcA, and a transcription activator, XpsR,

are most crucial (Genin and Denny 2012). At high cell density, *R. solanacearum* detects the autoinducer signal molecule, 3-OH palmitic acid methyl ester (3-OH PAME) and increases the level of functional PhcA. PhcA then activates the production of multiple virulence factors while suppressing survival and invasion related functions of the bacterium (Denny 2006). PhcA binds to the promoter region of *xpsR*, which enhances its expression and XpsR further enhances the expression of genes involved with EPS production. Recent studies have demonstrated that the quorum-sensing response is similar both in culture and in plant host stems (Kang et al. 1999; Jacob et al. 2012).

Apart from EPS production, *R. solanacearum* must come in contact with the host rhizosphere to initiate the infection with flagellar motility (Tans-Kersten et al. 2001). Non-motile mutants have significantly reduced ability to cause disease on tomato when inoculated through biologically representative soil drench methods. Once inside a susceptible host plant, bacterial population increases and develops biofilms in the host xylem (Tran et al. 2016). *R. solanacearum* multiply rapidly in the nutrient-poor xylem. Part of this ability may be attributed to the metabolic capacity of the bacteria, which utilizes organic and amino acids efficiently (Zuluaga et al. 2013; Peyraud et al. 2016), and its ability to adapt the oxygen-limited condition in the xylem as the bacterial population increases (Dalsing et al. 2015). These physiological insights about the pathogen provide a good framework from which to evaluate the efficacy of novel chemical compounds for control of bacterial wilt disease.

The present study was carried out to evaluate and compare *in vitro* activity of 3-indolylacetonitrile (IAN), 6,7-dihydroxycoumarin (6-7-D), and p-Benzoquinone (pBQ) against *R. solanacearum*. Specifically, we examined the effect of the compounds on *i*) inhibiting motility of the bacteria; *ii*) production of EPS by *R. solanacearum*; *iii*) inhibition of metabolizing various

sole carbon and nitrogen sources using the Biolog phenotype microarray; and *iv*) level of bacterial wilt severity in tobacco plants treated with these compounds under greenhouse conditions.

2. Material and methods

***2.1 Ralstonia solanacearum* strains and growth conditions.** *Ralstonia solanacearum* strain W7, previously characterized as phylotype IIA sequevar 7 (Tseng and Mila 2012), was isolated on 2,3,5-triphenyltetrazolium chloride (TZC) medium (Kelman 1953) from a diseased tomato stem collected in North Carolina in 2011 and stored in 15% glycerol at -80ºC. For routine culturing and preparation of inoculum, the bacterium was streaked on BG agar medium (1% Bacto peptone, 0.1% casamino acid, 0.1% yeast extract, 0.5% glucose, and 1.6% agar) and then incubated at 28 ºC in dark immediately after removing from the -80ºC glycerol stock.

2.2 Preparation of compound stock solutions. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) was used to prepare stock solutions of 3-indolylacetonitrile (IAN), 6,7-dihydroxycoumarin (6-7-D), and p-Benzoquinone (pBQ) (Sigma-Aldrich, St. Louis, MO) since the compounds are not water soluble. Stock solutions at 64mM for IAN, and 10mM for 6-7-D and pBQ were filter sterilized and stored at -20 ºC.

2.3 Motility assay. The effects of the compounds on bacterial motility were assayed on semi-solid BG media containing 0.2% (w/v) agar. The semi-solid media were amended with the compounds at a series of concentrations. Plates were inoculated with a 2 -µl drop of bacterial culture containing $1x10^8$ CFU/ml. Motility was visualized as a white halo of cells moving outward from the original inoculation site after 2 and 4 days of incubation at 25°C.

2.4 Extracellular polysaccharide quantification. The amount of extracellular polysaccharide (EPS) in culture broth supernatant was determined by measuring hexoamine content with the Elson-Morgan biochemical Assay (Peyraud et al. 2017). Briefly, EPS in culture supernatant was precipitated by adding NaCl at 0.1 mM final concentration and 4 volumes of acetone then stored at 4°C over night. The precipitated EPS was resuspended in sterilized dH_2O and heated at 65°C for 10 min, then removing the insoluble material by centrifugation at 13,000 rpm for 5 min. For 200 µl of EPS sample, 150 µl of 12M HCl and 250 µl dH_2O were added and heated at 110 °C for 30 min to hydrolyze the sugar polymers. The hydrolyzed samples were then slowly mixed with 400 µl of 2 M Na_2CO_3 and 500 µl of 2% acetyl acetone in 1.5 M Na2CO_3, and heated at 100 °C for 20 min. After cooling to room temperature, the liquid was transferred into 15 ml Falcon tubes and mixed with 1.0 ml of 95% ethanol. Five-hundred µl of Erlich's reagent solution (0.4 g of para-dimethyl-aminobenzaldehyde in 6.0 ml ethanol and 6.0 ml 12M HCl) was added. The samples were kept for 30 min in the dark and then the absorbance at 530 nm was recorded. Quantification of hexosamine in the samples was determined by comparing with standard curve of N-acetyl-galactosamine which followed the overall process except precipitation.

2.5 Phenotype microarray assay. The effect of the compounds on bacterial carbon and nitrogen metabolism were examined by using Biolog Phenotype Microarray plates, PM1 and 3, following the manufacturer's protocol. PM1 tests for the bacterium's ability to metabolize specific substrate as sole carbon source; PM3 tests for nitrogen source. Briefly, *R. solanacearum* cells were collected from a static culture on a BG plate and resuspended in Biolog fluid IF-0 and adjusted to OD_{600} = 0.37. The compounds were added to the IF-0 bacterial suspension using sub-minimal inhibition concentrations (MICs) (IAN at 20 µM pBQ at 12.5 µM, and 6-7-D at 50 µM)

immediately before dispensing into the assay plates. The plates were incubated at 28°C and measurements were recorded on Omnilog reader (Biolog) for 96 hours. Data were analyzed by calculating the area under curve then scored 0: < 5% increase compared to control; 1: ≥ 5%, < 20%; 2: ≥ 20%.

2.6 RNA extraction and quantitative real-time PCR analyses. To measure gene expression of bacteria growing in culture, RNA was extracted from 1-ml cultures of *R. solanacearum* grown in minimal medium (MM) (Clough et al. 1994) amended with 0.01% (w/v) casamino acids and 1.5% (w/v) filter-sterilized glucose. To initiate the broth culture, 1 x 10^5 cells from freshly grown CPG plates were cultured in MM with casamino acid and glucose shaken (250 rpm) at 28°C. The cultures were incubated for 18 h then treated with the compounds at desired concentrations (IAN at 20 µM, pBQ at 12.5 µM, and 6-7-D at 50 µM) for additional 6 hours. After incubation, 1-ml cultures were used for RNA extraction. Briefly, bacterial cells were harvested by centrifugation at 8000 rpm for 1 min. Cells were disrupted by bead beating for 20 s then immediately adding 1 ml of TRIzol® reagent (Thermo Fisher Scientific, Waltham, MA.) The RNA was then isolated per the manufacturer's instructions. The RNA samples were reverse-transcribed using ProtoScript® II reverse transcriptase (New England Biolabs, Beverly, MA).

Quantitative real-time PCR analyses were carried out in 96-well plates in an Applied BioSystems QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) with iTaq Universal SYBR green Supermix (BioRad, Hercules, CA). The amplification program was as follows: 30 sec at 95°C; 40 cycles of 95°C for 15 sec, 60°C for 30 sec. Oligonucleotides used as primers were synthesized by Integrated DNA technology (Coralville, IA) and indicated in Table 1. Three biological and three technical replicate reactions were used for each sample. Comparative C_T method was used to analyze the real-time PCR data

(Schmittgen and Livak, 2008) normalizing gene expression with two reference genes, *serC* and *rplM* (Monteiro et al. 2012).

2.7 Greenhouse assay. Tobacco cultivars K326 and Speight168 were used. Tobacco plants were grown from seed directly in 3.5-by-3.5-cm cell flats filled Fafard 4M potting mix (Fafard Inc., Agawam, MA) (1:1:1) and fertilized 3 weeks after seeding. Each plant was supplied with 200 ppm water soluble fertilizer (20-10-20, N-P-K). The plants were transplanted to 4-inch pots filled with a mixture of soil, sand and Fafard 4M potting mix (Fafard Inc., Agawam, MA) (1:1:1) 4 weeks after seeding.

Inoculation was conducted when tobacco plants were 6 weeks old by stabbing a sterilized scalpel around each seedling then immediately pouring a bacterial suspension onto the soil to a final density of around $3x10^7$ CFU/g of soil. There were three replications of five plants per treatment. Plants were treated with IAN at 31.25 µg, pBQ at 6.75 µg, or 6-7-D at 55.67 µg, respectively, at the same time that plants were inoculated by drenching the soil with 50 ml dH_2O. Non-inoculated plants were used as the negative and inoculated non-treated as positive controls. Plants were rated daily for disease severity using a modified Horsfall-Barratt index scale: 0: no leaves wilted; 1: 1%–25% leaves wilted; 2: 26%–50% leaves wilted; 3: 51%–75% leaves wilted; 4: >75% leaves wilted (Tans-Kersten et al., 2001). The experiment was repeated twice, with 15 plants per treatment per biological replicate.

3. Results

3.1 IAN and 6-7-D inhibit *R. solanacearum* motility *in vitro*. Measurement of the motility plates displayed a significantly ($P<0.0001$) reduced migration from the inoculation site to the periphery of the plate when bacteria was tested on motility assay plates amended IAN or

6-7-D, but not pBQ when compared to untreated control (Figure 1). In untreated control, the bacteria were able to cover the plate (8.5 cm) in 4 days. When tested on plates amended with IAN, the halo indicating bacterial migration was significantly ($P<0.0001$) smaller (3.0 cm) even at the lowest concentration tested (10 µM). The inhibitory effect increased as IAN concentration increased to 20 and 40 µM. At 80 µM, IAN inhibited the bacterial growth completely. The inhibitory effects of 6-7-D on bacterial motility was significant at 100 and 125 µM. pBQ did not inhibit *R. solanacearum* motility at all four concentration.

3.2 IAN and 6-7-D trigger EPS production in *R. solanacearum* cells. EPS is a major virulence factor of *R. solanacearum* and provides protection from a wide range of environmental stresses. The Elson-Morgan biochemical Assay was used to quantify EPS produced by comparable numbers (10^8 CFU) of *R. solanacearum* cells growing in MM amended with the compounds (20 µM of IAN, 12.5 µM of pBQ, or 50 µM of 6-7-D). After exposing to IAN or 6-7-D for 6 h, *R. solanacearum* produced significantly more EPS when compared to cells grown in untreated control (Fig. 2). Exposing to pBQ did not significantly affect the amount of EPS produced by *R. solanacearum*.

3.3 Differential gene expression of *R. solanacearum* cells with the compounds. To better understand the mechanisms by which IAN and 6-7-D inhibited motility and enhanced EPS production, qRT-PCR was used to evaluate the expression of important regulators in *R. solanacearum*. The selected genes included the quorum-sensing response regulator *phcA*, EPS production and virulence regulator *xpsR*, response regulator *pehR* which controls flagellar motility, and chemotaxis histidine autokinase *cheA*. In addition, the expression of type III secretion regulator, *hrpB*, was also included as it plays a critical role in disease development. All three compounds increased the expression of *phcA* about 0.5 fold compared to untreated control

(Figure 3). IAN and 6-7-D increased the expression of *xpsR* by 1.5 fold and 0.5 fold respectively, but pBQ did not have significant effect of *xpsR* expression when compared with untreated control. The increased expression of *xpsR* result corresponds to our EPS quantification assay results. IAN and 6-7-D increased the expression of *cheA* by 2 fold and 1.5 fold respectively, while pBQ did not have significant effect. The expression level of *pehR* was increased by 0.5 fold when cells were treated with IAN, while pBQ reduced its expression by 0.5 fold. 6-7-D did not have a significant effect on *pehR* expression. The expression level of type III secretion system regulator, *hrpB*, was only reduced by IAN but not pBQ or 6-7-D.

3.4 IAN reduced wilting symptoms in susceptible tobacco cultivar K326. To evaluate the compounds' ability to reduce disease development on tobacco plants, tobacco cultivar K326 (susceptible) and Speight 168 (moderately resistant) were used for disease severity assays. On cultivar K326, untreated plants started to show symptoms of leaf wilting 6 days after inoculation and were completely wilted within 20 days of the experiments. When treated with 31.25 μg of IAN at the time of inoculation, disease severity was significantly less than untreated control at the end of the experiments (Figure 4). When treated with pBQ at 6.75 μg, or 6-7-D at 55.67 μg, the disease severity was not significantly different compared to untreated controls on cultivar K326. With cultivar Speight 168, wilting symptoms on untreated plants started to appear 8 days after inoculation. None of the treatments delayed the symptom initiation. After 20 days, treating the plants with pBQ at 6.75 μg resulted a lower disease index when compared to untreated control. IAN and 6-7-D did not significantly reduce disease severity on moderately resistant cultivar Speight 168.

3.5 Effects of the compounds on *Ralstonia solanacearum* utilization of various carbon sources. Among the 95 carbon substrates tested, *R. solanacearum* was able to utilize 35

as sole carbon source (Table 2). When treated with the compounds, no additional carbon substrate became available to the bacterium (Table 3). IAN inhibited the ability of *R. solanacearum* to metabolize different carbon substrates, reducing the number of utilized carbon substrates to five (L-glutamic acid, Tween 20, L-asparagine, L-glutamine, and methylpyruvate). In addition, all five substrates were utilized in a very limited manner (< 20% increase compared to negative control).

pBQ reduced the number of carbon substrates utilized by *R. solanacearum* from 35 to 29, and overall metabolism of carbon substrates was reduced. Specifically, when treated with pBQ, utilization of 13 carbon substrates was inhibited (score of 2, ≥ 20% increase compared to negative control) in untreated cultures to a score of 1. The utilization of D-alanine, glycerol, acetic acid, α-ketoglutaric acid, Tween 80, and propionic acid were scored 0 (< 5% increase compared to negative control), indicating strong inhibition (Table 3). Among the 6 substrates, except for Tween 80, five were scored 1 in untreated cultures, indicating that they were not used efficiently in *R. solanacearum*, and pBQ further inhibited the utilization of these substrates.

6-7-D reduced the number of carbon sources utilized by the bacterium from 35 to 24. Seven substrates (L-aspartic acid, D-trehalose, L-glutamic acid, Tween 40, Tween 80, m-inositol, and mucic acid) that were scored 2 in untreated culture were reduced to 1 when treated with 6-7-D. The utilization of D-saccharic acid and D-glucuronic acid were reduced from 2 in untreated culture to 0 when treated with 6-7-D (Table 3). These results suggest that 6-7-D may have a direct effect on utilization of these two carbon substrates as sole carbon source.

3.6 Effects of the compounds on *Ralstonia solanacearum* utilization of various nitrogen sources. *Ralstonia solanacearum* utilized 37 of the tested nitrogen substrates. When

treated with the compounds, the bacterium's ability to utilize the nitrogen substrates showed various changes.

When treated with IAN, the total number of utilized nitrogen substrate reduced from 37 to 30 (Table 2). There were eight substrates with score of 0 that were scored 1 or 2 in untreated cultures, while one substrate (methylamine) was scored 1 instead of 0 in untreated culture. In addition, seven substrates received a score of 2 when treated with IAN instead of 1 in untreated culture (Table 4).

When treated with pBQ, utilization of many nitrogen substrates showed an increase. Specifically, 14 substrates that were scored 0 in untreated culture increased to a score of 2 when treated with pBQ, for example, nitrite, nitrate, urea, inosine, xanthine, and some di-peptide substrates. Ten substrates that were scored 1 in untreated culture increased to a score of 2 when treated with pBQ. No substrate showed reduced utilization (Table 4).

6-7-D also did not show any inhibitory effects on *R. solanacearum* nitrogen utilization. Instead, 13 substrates that were scored 1 in untreated culture increased to a score of 2 when treated with 6-7-D. Seven substrates that were scored 0 in untreated culture increased to a score of 1 or 2 when treated with 6-7-D. The full list of nitrogen substrates tested in the Biolog assay plates can be found at Table 4.

4. Discussion

This study examined how chemical compounds may affect multiple aspects of bacterial physiology and reduce disease severity. Among the three compounds examined in this study, IAN had the greatest effect on both interfering with bacterial physiological functions and reducing disease severity. IAN also reduced motility of *R. solanacearum* at a concentration

without affecting its growth. In addition, the expression of two important regulators involved with virulence, *phcA* and *xpsR*, were increased when treated with IAN in broth culture. This appears counterintuitive to disease reduction as these two genes are highly expressed when *R. solanacearum* is inside host xylem (Jacobs et al. 2012). One possible explanation is that the changes in gene expression may be a result of reduced motility. The expression of *phcA* is regulated through quorum-sensing mechanism and the signal molecule 3-hydroxy palmitic acid methyl ester (3-OH PAME). As cell density increases, 3-OH PAME produced by *R. solanacearum* cells accumulates in the extracellular environment. The bacterial cells respond to the threshold concentration of 3-OH PAME and increases the level of functional PhcA. PhcA then activates the production of virulence factors, including extracellular polysaccharide (EPS), through transcriptional activation of *xpsR* and other genes (Genin and Denny, 2012). Previous research has demonstrated the importance of motility in early stages of host plant invasion and colonization (Tans-Kersten et al. 2001) but did not describe how non-motile mutants behave in terms of quorum-sensing-related virulence factors. When motility is inhibited, bacterial cells grown in broth culture form microcolonies as they multiply. The quorum-sensing signaling may be affected within the microcolonies thus triggered higher level of *phcA* expression when compared with the untreated control. The effect of 6-7-D on the physiology and virulence of *R. solanacearum* shares some similar features to IAN. Although to lesser degree, 6-7-D also inhibited *R. solanacearum* motility on semi-solid medium plates and increased the expression of *phcA* and *xpsR*. This may be another indication of inhibition of bacterial motility affects quorum-sensing signaling and related gene expression. However, without affecting motility, pBQ also increased the expression level of *phcA*. The cause for these physiological changes cannot be

determined definitively without further understanding how the compounds affect cellular functions at a molecular level.

The qRT-PCR results could not explain the inhibition of bacterial motility when treated with the IAN or 6-7-D. Previous research had shown that in *R. solanacearum*, *pehR* is negatively regulated by *phcA* and inactivation of *pehR* causes loss of motility (Allen et al. 1997). When bacterial cells were treated with IAN, *phcA* and *pehR* expression levels were increased by 0.5 fold compared to the untreated control, even though motility was significantly inhibited. This suggests that the inhibition in motility may be through other mechanisms, for example, structural or functional disruption of the flagella. Unfortunately, after multiple attempts of examining treated bacterial cells using electron microscopy, the presence or absence of flagella in treated bacterial cells could not be confirmed (data not shown). Previous results have demonstrated that IAN greatly reduces *R. solanacearum* colonization on tobacco roots. Our results indicated that IAN increases the expression level of *cheA*, the chemotaxis response regulator. Inhibited motility or interfering with bacterial chemotaxis could be the reason for the reduced root colonization.

IAN greatly inhibited the ability of *R. solanacearum* to utilize various substrates as sole carbon source, particularly, sucrose, glutamic acid, aspartic acid, and asparagine (Table 3). The plant xylem is considered a nutrient-limiting, low-oxygen environment (Pegg 1985). Under normal conditions, tomato xylem sap composition includes no detectable amounts of glucose or fructose (Hiery et al. 2013). Organic and amino acids, relatively abundant in xylem fluid, were considered primary carbon sources for *R. solanacearum* during xylem infection (Bialczyk and Lechowski, 1995; Brown and Allen, 2004). Previous *in planta* transcriptome studies of *R. solanacearum* have shown that sucrose uptake and catabolism was highly expressed during pathogenesis and required for full virulence (Jacobs et al. 2012). Being able to use available

nutrient sources is crucial for disease development. The exact mechanism of IAN inhibiting carbon metabolism requires further investigation, and inhibiting the ability of *R. solanacearum* to metabolize these carbon substrates may play a role in reducing disease severity.

The mechanism for IAN to reduce disease severity on susceptible tobacco cultivar K326 may be a combination of multiple factors throughout disease development. A recent study had demonstrated that *R. solanacearum* produces biofilm in the host xylem, and the modulation of their biofilm structure is important for systemic spread and virulence (Tran et al. 2016). The biofilm matrix, in which the bacterial cells are embedded, consists of polysaccharides, proteins and DNA (Flemming and Wingender 2010; Montanaro et al. 2011). An *R. solanacearum* mutant lacking the extracellular nucleases facilitating biofilm maturation and bacterial dispersal formed non-spreading colonies and abnormally thick biofilms *in vitro*. This mutant was also reduced in virulence on tomato plants and did not spread in tomato stems as well as the wild-type strain (Tran et al. 2016). Enhanced biofilm and EPS production when treated with IAN limited the bacteria spread in host stem, which may be a possible explanation for the reduced disease severity observed. Further research is needed to accept this hypothesis.

On the moderately resistant tobacco cultivar, Speight 168, IAN did not significantly reduce disease severity when compared to the untreated controls. The difference in effectiveness of the compound between susceptible and moderately resistant cultivar is interesting. In tomato, where the mechanism of resistance has been studied extensively, resistance was associated with the ability of plants to limit bacterial colonization of the vascular tissues of the stem (Grimault and Prior 1993; Prior et al. 1996). Lebeau et al. (2011) investigated several genetic resources of tomato, pepper, and eggplant and concluded that, in addition to limitation of bacterial colonization in vascular elements, resistance mechanism also lays in plant survivability despite

the presence of bacteria in the vessels. Despite the lack of resistance to bacterial wilt, K326 is still one of the most popular cultivars planted in North Carolina (Fisher et al. 2018). Bacterial wilt management will always require a combination of cultural practices, host resistance, and chemical control. Instead of simply looking for antimicrobial compounds based on growth inhibition *in vitro*, this research demonstrates an example of how utilizing the knowledge of bacterial physiology and pathogenicity can give researchers valuable criteria for evaluating potential chemical agents.

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Table 1. Oligonucleotides used in this study.

Gene target	Primer name	Primer sequences (5' → 3')	Reference
serC	serC-F	GGATGACGCGGCTTACGT	Monteiro et al. 2012
	serC-R	TCAACGCCGACGATGGT	
rplM	rplM-F	CCGCAAAGCCCCATGAG	Monteiro et al. 2012
	rplM-R	TGTCCGTCGCGTCAATCA	
phcA	phcA-F	GTCGTCACCTCCTTCAACAT	This study
	phcA-R	TGAAGATATGCTCGCACTCG	
cheA	cheA-F	CATGATGCCGATGGACTATGT	This study
	cheA-R	GTCGGTTTGCCGAAGGT	
pehR	pehR-F	TGCTGCTGGAGCAACTG	This study
	pehR-R	GAACGGATAGGCGCACAA	
xpsR	xpsR-F	ATGGCAATCGCACCAGAA	This study
	xpsR-R	AGCAGATCCGCGAAGTAATG	
hrpB	hrpB-F	GGAAAGTCCGACGACTACGC	Monteiro et al. 2012
	hrpB-R	TCTTCATCGCACTCGAGCAG	Monteiro et al. 2012

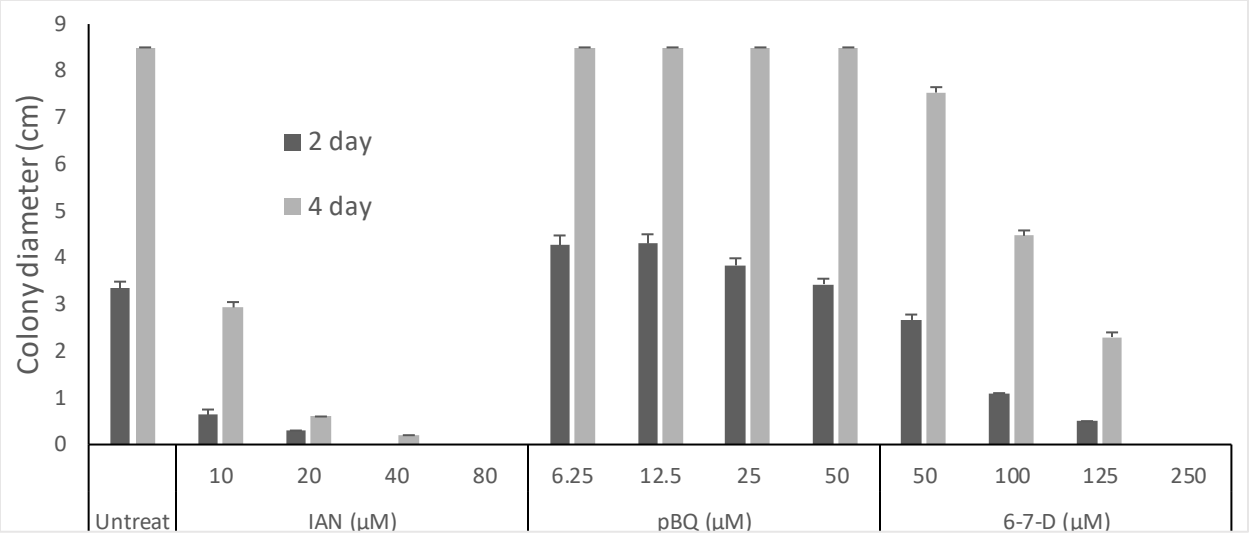

Figure 1. Motility assay in BG soft (0.2%) agar plates amended with the compounds at various concentration. Bars represents the diameter measurements of the halos formed by the different treatments after 2 and 4 days. Error bars represent the standard error. The experiment was repeated twice.

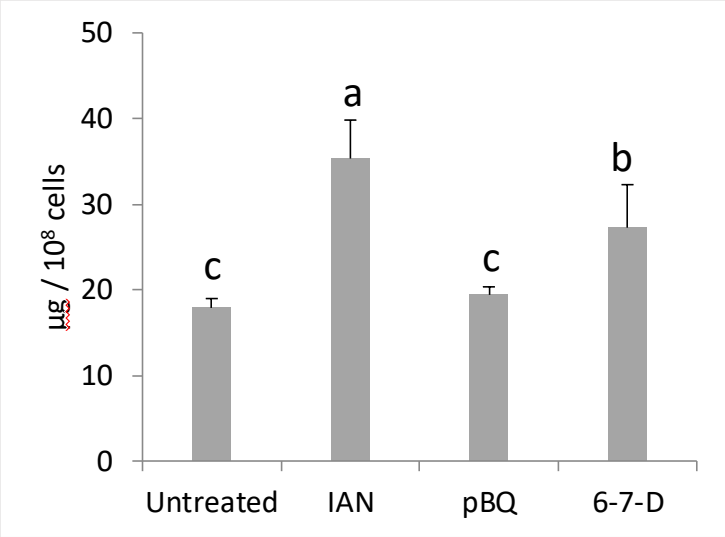

Figure 2. Quantification of EPS in culture supernatant using Elson-Morgan assay. *Ralstonia solanacearum* strain W7 was grown in minimal medium for 18 h, then the compounds (20 µM of IAN, 12.5 µM of pBQ, or 50 µM of 6-7-D) were added and the incubation was continued incubation for an additional 6 h prior to the assay. Error bars represent the standard error. Different letters indicate significant differences (P = 0.05) among treatments. The experiment was repeated twice.

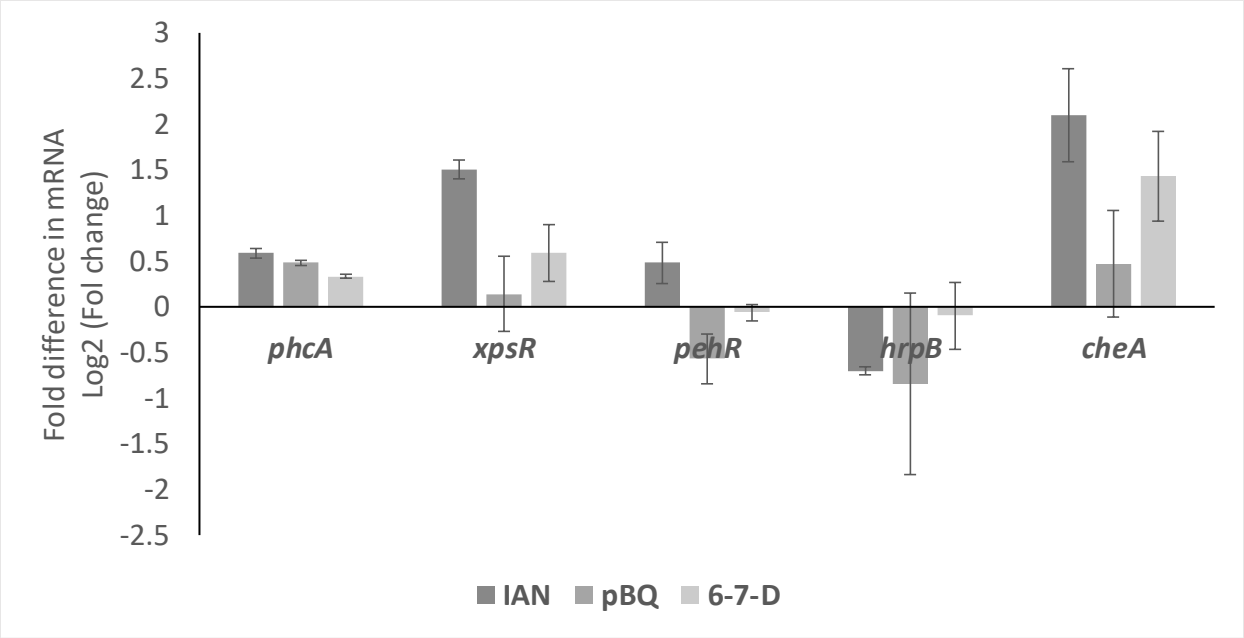

Figure 3. Differential gene expression in *R. solanacearum* strain W7 cells grown in minimal medium for 18 h, then the compounds (20 μM of IAN, 12.5 μM of pBQ, or 50 μM of 6-7-D) were added and the incubation continued for an additional 6 h prior to qRT-PCR analysis. Data were presented as the ratio of transcript number in the treatment of the compounds with that in the untreated control. *serC* and *rplM* were used as endogenous control. Three biological and three technical replicate reactions were used for each treatment.

Figure 4. Disease progress of *R. solanacearum* when treated with the compounds at inoculation. Six-week-old tobacco plants (cv. K326 and Speight 168) were inoculated by pouring bacterial suspensions near the crown immediately after wounding the roots to a final concentration of $3x10^7$ CFU/g of soil. The compounds (IAN at 31.25 µg, pBQ at 6.75 µg, or 6-7-D at 55.67 µg) were mixed in the bacterial suspension immediately before inoculation. Plants were rated every two days in the first 8 days then rated daily to 20 days on a disease index scale from 0 to 4. Each point represents the mean disease index of three individual experiments each containing 15 plants per treatment.

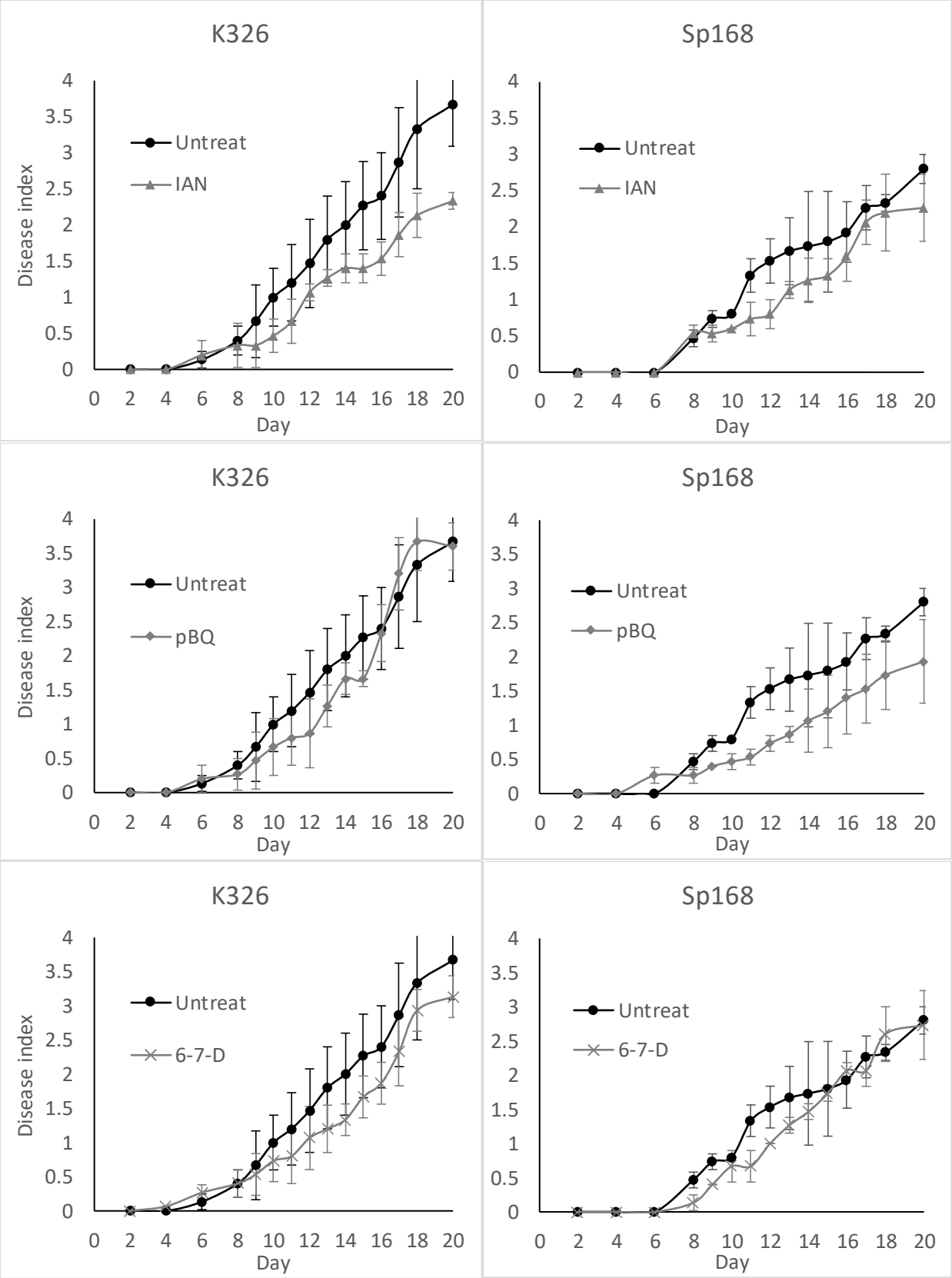
K326
Sp168
Disease index
Day
Untreat
IAN
pBQ
6-7-D
0
0.5
1
1.5
2
2.5
3
3.5
4
0 2 4 6 8 10 12 14 16 18 20

Table 2. Effects of the compounds on *Ralstonia solanacearum* utilization of various carbon and nitrogen sources

Biolog Plate	Treatment [a]	Score [b]	No. of substrates
PM1	Untreated	0	60
Carbon		1	14
Sources		2	21
	IAN	0	89
		1	6
		2	0
	pBQ	0	66
		1	21
		2	8
	6-7-D	0	71
		1	13
		2	11
PM3	Untreated	0	58
Nitrogen		1	14
Sources		2	23
	IAN	0	65
		1	3
		2	27
	pBQ	0	42
		1	6
		2	47
	6-7-D	0	48
		1	5
		2	42

[a] The treatments were 3-indolylacetonitrile (IAN, 20 μM), 6,7-dihydroxycoumarin (6-7-D, 50 μM), and *p*-Benzoquinone (pBQ, 12.5 μM).

[b] Data were analyzed by calculating the area under curve then scored 0: < 5% increase compared to control; 1: ≥ 5%, < 20%; 2: ≥ 20%.

Table 3. List of substrates and scoring when treated with the compounds for PM1 assay.

	Treatments [a]			
Substrate	**Untreated**	**IAN**	**pBQ**	**6-7-D**
L-Arabinose	0 [b]	0	0	0
N-Acetyl-D-Glucosamine	0	0	0	0
D-Saccharic acid	2	0	1	0
Succinic acid	2	0	1	2
D-Galactose	0	0	0	0
L-Aspartic acid	2	0	1	1
L-Proline	0	0	0	0
D-Alanine	1	0	0	0
D-Trehalose	2	0	1	1
D-Mannose	0	0	0	0
Dulcitol	0	0	0	0
D-Serine	0	0	0	0
D-Sorbitol	0	0	0	0
Glycerol	1	0	0	0
L-Fucose	0	0	0	0
D-Glucuronic acid	2	0	1	0
D-Gluconic acid	0	0	0	0
DL-a-Glycerol Phosphate	0	0	0	0
D-Xylose	0	0	0	0
L-Lactic acid	0	0	0	0
Formic acid	0	0	0	0
D-Mannitol	0	0	0	0
L-Glutamic acid	2	1	2	1
D-Glucose-6-Phosphate	0	0	0	0
D-Galactonic acid-g-Lactone	0	0	0	0
DL-Malic acid	1	0	1	1
D-Ribose	0	0	0	0
Tween 20	2	1	1	2
L-Rhamnose	0	0	0	0
D-Fructose	1	0	1	0
Acetic acid	1	0	0	0

Table 3. (continued) List of substrates and scoring when treated with the compounds for PM1 assay.

	Treatments			
Substrate	**Untreated**	**IAN**	**pBQ**	**6-7-D**
a-D-Glucose	1	0	1	1
Maltose	0	0	0	0
D-Melibiose	0	0	0	0
Thymidine	0	0	0	0
L-Asparagine	2	1	1	2
D-Aspartic acid	0	0	0	0
D-Glucosaminic acid	0	0	0	0
1,2-Propanediol	0	0	0	0
Tween 40	2	0	1	1
a-Ketoglutaric acid	1	0	1	1
a-Ketobutyric acid	1	0	0	0
a-Methyl-D-Galactoside	0	0	0	0
a-D-Lactose	0	0	0	0
Lactulose	0	0	0	0
Sucrose	1	0	1	0
Uridine	0	0	0	0
L-Glutamine	2	1	2	2
m-Tartaric acid	0	0	0	0
D-Glucose-1-Phosphate	0	0	0	0
D-Fructose-6-Phosphate	0	0	0	0
Tween 80	2	0	0	1
a-Hydroxyglutaric acid-g-Lactone	0	0	0	0
a-Hydroxybutyric acid	0	0	0	0
b-Methyl-D-Glucoside	0	0	0	0
Adonitol	0	0	0	0
Maltotriose	0	0	0	0
2`-Deoxyadenosine	0	0	0	0
Adenosine	0	0	0	0
Gly-Asp	0	0	0	0
Citric acid	2	0	2	2

Table 3. (continued) List of substrates and scoring when treated with the compounds for PM1 assay.

	Treatments			
Substrate	**Untreated**	**IAN**	**pBQ**	**6-7-D**
m-Inositol	2	0	1	1
D-Threonine	0	0	0	0
Fumaric acid	2	0	2	1
Bromosuccinic acid	1	0	1	0
Propionic acid	1	0	0	0
Mucic acid	2	0	1	1
Glycolic acid	0	0	0	0
Glyoxylic acid	0	0	0	0
D-Cellobiose	0	0	0	0
Inosine	0	0	0	0
Gly-Glu	0	0	0	0
Tricarballylic acid	0	0	0	0
L-Serine	1	0	1	1
L-Threonine	1	0	1	1
L-Alanine	2	0	2	2
Ala-Gly	0	0	0	0
Acetoacetic acid	0	0	0	0
N-Acetyl-D-Mannosamine	0	0	0	0
Mono-Methylsuccinate	1	0	1	0
Methylpyruvate	2	1	2	2
D-Malic acid	0	0	0	0
L-Malic acid	2	0	2	2
Gly-Pro	0	0	0	0
p-Hydroxyphenyl Acetic acid	0	0	0	0
m-Hydroxyphenyl Acetic acid	0	0	0	0
Tyramine	0	0	0	0
D-Psicose	0	0	0	0
L-Lyxose	0	0	0	0
Glucuronamide	0	0	0	0
Pyruvic acid	2	1	2	2

Table 3. (continued) List of substrates and scoring when treated with the compounds for PM1 assay.

	Treatments			
Substrate	**Untreated**	**IAN**	**pBQ**	**6-7-D**
L-Galactonic acid-g-Lactone	2	0	1	2
D-Galacturonic acid	2	0	1	2
Phenylethylamine	0	0	0	0
2-Aminoethanol	0	0	0	0

[a] The treatments were 3-indolylacetonitrile (IAN, 20 μM), 6,7-dihydroxycoumarin (6-7-D, 50 μM), and *p*-Benzoquinone (pBQ, 12.5 μM).

[b] Data were analyzed by calculating the area under curve then scored 0: < 5% increase compared to control; 1: ≥ 5%, < 20%; 2: ≥ 20%.

Table 4. List of substrates and scoring when treated with the compounds for PM3 assay.

	Treatments [a]			
Substrate	**Untreated**	**IAN**	**pBQ**	**6-7-D**
Ammonia	2 [b]	0	2	2
Nitrite	0	0	2	0
Nitrate	0	0	2	0
Urea	0	0	2	0
Biuret	0	0	0	0
L-Alanine	2	2	2	2
L-Arginine	1	0	2	1
L-Asparagine	1	2	2	2
L-Aspartic acid	1	2	2	2
L-Cysteine	0	0	2	1
L-Glutamic acid	2	2	2	2
L-Glutamine	2	2	2	2
Glycine	0	0	0	0
L-Histidine	2	2	2	2
L-Isoleucine	2	2	2	2
L-Leucine	2	2	2	2
L-Lysine	1	0	0	2
L-Methionine	2	2	1	2
L-Phenylalanine	2	2	2	2
L-Proline	1	2	2	2
L-Serine	1	2	1	2
L-Threonine	2	2	2	2
L-Tryptophan	2	2	2	2
L-Tyrosine	2	2	2	2
L-Valine	2	1	2	2
D-Alanine	2	2	2	2
D-Asparagine	2	2	2	2
D-Aspartic acid	2	2	2	2
D-Glutamic acid	0	0	0	0
D-Lysine	0	0	0	0
D-Serine	0	0	0	0

Table 4. (continued) List of substrates and scoring when treated with the compounds for PM3 assay.

	Treatments [a]			
Substrate	**Untreated**	**IAN**	**pBQ**	**6-7-D**
D-Valine	0	0	0	0
L-Citrulline	2	2	2	2
L-Homoserine	0	0	0	0
L-Ornithine	0	0	2	0
N-Acetyl-L-Glutamic acid	0	0	1	0
N-Phthaloyl-L-Glutamic acid	0	1	2	2
L-Pyroglutamic acid	1	2	2	2
Hydroxylamine	0	0	0	0
Methylamine	0	1	1	2
N-Amylamine	0	0	2	1
N-Butylamine	0	0	0	2
Ethylamine	0	0	0	2
Ethanolamine	0	0	0	1
Ethylenediamine	0	0	0	0
Putrescine	0	0	0	0
Agmatine	0	0	0	0
Histamine	0	0	0	0
b-Phenylethylamine	0	0	0	0
Tyramine	0	0	0	0
Acetamide	0	0	0	0
Formamide	0	0	0	0
Glucuronamide	2	2	2	2
DL-Lactamide	0	0	0	0
D-Glucosamine	0	0	0	0
D-Galactosamine	0	0	0	0
D-Mannosamine	0	0	0	0
N-Acetyl-D-Glucosamine	0	0	0	0
N-Acetyl-D-Galactosamine	0	0	0	0
N-Acetyl-D-Mannosamine	0	0	0	0
Adenine	0	0	0	0

Table 4. (continued) List of substrates and scoring when treated with the compounds for PM3 assay.

	Treatments [a]			
Substrate	**Untreated**	**IAN**	**pBQ**	**6-7-D**
Adenosine	0	0	0	0
Cytidine	0	0	0	0
Cytosine	0	0	0	0
Guanine	2	2	2	2
Guanosine	1	0	1	2
Thymine	0	0	0	0
Thymidine	0	0	0	0
Uracil	0	0	0	0
Uridine	0	0	0	0
Inosine	0	0	2	0
Xanthine	0	0	2	1
Xanthosine	0	0	0	0
Uric acid	1	2	2	2
Alloxan	1	0	2	2
Allantoin	0	0	2	0
Parabanic acid	0	0	0	0
DL-a-Amino-N-Butyric acid	0	0	0	0
g-Amino-N-Butyric acid	1	2	2	2
e-Amino-N-Caproic acid	1	0	1	2
DL-a-Amino-Caprylic acid	0	0	0	0
d-Amino-N-Valeric acid	0	0	0	0
a-Amino-N-Valeric acid	0	0	0	0
Ala-Asp	0	0	0	0
Ala-Gln	2	2	2	2
Ala-Glu	2	2	2	2
Ala-Gly	0	0	2	2
Ala-His	1	0	2	2
Ala-Leu	0	0	2	2
Ala-Thr	1	0	2	2
Gly-Asn	2	0	2	2

Table 4. (continued) List of substrates and scoring when treated with the compounds for PM3 assay.

	Treatments [a]			
Substrate	**Untreated**	**IAN**	**pBQ**	**6-7-D**
Gly-Gln	2	2	2	2
Gly-Glu	0	0	2	0
Gly-Met	0	0	2	0
Met-Ala	0	0	2	0

[a] The treatments were 3-indolylacetonitrile (IAN, 20 μM), 6,7-dihydroxycoumarin (6-7-D, 50 μM), and *p*-Benzoquinone (pBQ, 12.5 μM).

[b] Data were analyzed by calculating the area under curve then scored 0: < 5% increase compared to control; 1: ≥ 5%, < 20%; 2: ≥ 20%.