DISSERTATION

UNDERSTANDING THE IMPACT OF RUSSIAN WHEAT APHID-ASSOCIATED MICROBES ON PLANTS

Submitted by

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ABSTRACT

UNDERSTANDING THE IMPACT OF RUSSIAN WHEAT APHID-ASSOCIATED MICROBES ON PLANTS

Plant-insect-microbe interactions shape the outcome of plant defense responses, yet the role of aphid-associated bacteria in modulating host susceptibility remains largely unexplored. This dissertation investigates the influence of bacterial communities associated with the Russian wheat aphid (RWA, *Diuraphis noxia*) on plant responses, focusing on barley and wheat as plant host systems. Through a combination of proteomics, microbiome analysis, transcriptomics and biological assays, this work provides novel insights into how aphid-associated microbes contribute to the amount of damage aphids cause to plants (aggressiveness) and alter plant hormone signaling.

In Chapter 2, proteomic and microbiome analyses reveal that the saliva of RWA is enriched with bacterial proteins, predominantly from Enterobacteriaceae. Several bacteria genera were isolated from aphids and wheat plants only after aphid feeding, suggesting horizontal transmission of microbes during infestation. Experimental reduction of bacterial populations in aphids led to decreased chlorosis in wheat, providing direct evidence that aphid-associated bacteria enhance aphid aggressiveness.

Chapter 3 characterizes a novel aphid-associated bacterial species, *Winslowiella iniecta* (previously, *Erwinia iniecta*) isolated from RWA and identified through multilocus sequence analysis and whole genome sequencing. While *W. iniecta* was not pathogenic to plants, its

presence influenced aphid performance, suggesting an indirect role in aphid fitness and plant interactions.

Chapter 4 demonstrates that honeydew is the primary source of aphid-acquired bacteria. Using RNA-sequencing and network analysis, we show that bacterial-enriched honeydew significantly alters barley defense pathways, particularly by modulating salicylic acid (SA) and jasmonic acid (JA) signaling. Barley plants infiltrated with honeydew from bacteria-rich aphid colonies exhibited stronger transcriptomic responses, characterized by enhanced SA activation alongside simultaneous JA suppression and degradation.

We purpose a model in which aphids acquire bacteria by feeding on honeydew-contaminated leaves. As they probe and feed, bacteria or bacterial proteins are introduced into the plant via the stylet, where they interact with plant cells, leading to SA upregulation and JA suppression.

Collectively, this dissertation advances our understanding of plant-aphid-microbe interactions, identifying bacterial contributions to aphid aggressiveness and demonstrating previously unrecognized role of honeydew in shaping plant defense responses to RWA. These findings have implications for the development of aphid management strategies, particularly in breeding crops with enhanced resilience to aphids.

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Chapter 1: INTRODUCTION

Background and Context

Aphid pests threaten global food security. Their feeding reduces crop yields, resulting in substantial economic losses due to the direct feeding damage and virus transmission. Using their specialized mouthparts, known as the stylet, aphids pierce plant tissues and navigate intercellularly (between cells) toward the sucrose-rich phloem, their primary food source. While doing so, they puncture cells (gaining intracellular access) to assess suitability for feeding. These stylet-induced wounds are rapidly sealed by gelling saliva, which allows aphids to feed with minimal activation of plant defense pathways that are typically triggered by chewing herbivores.

The Russian Wheat Aphid (*Diuraphis noxia*)

Russian wheat aphid (RWA, *Diuraphis noxia*) is a major pest of wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and other small grains. It is distributed worldwide, with recent expansions into Australia (Yazdani et al., 2018). Since its introduction into the United States (US) in the 1980s, RWA has caused over $1 billion in wheat losses (Mornhinweg et al., 2006; Quisenberry & Peairs, 1998). Although RWA causes direct feeding damage to the leaves and flowers, its main impact on plants is yellowing, stunted growth and loss of vigor, which significantly reduce grain yield. In severe cases, RWA infestations can result in up to 80% yield loss (Dedryver et al., 2010).

Management strategies for RWA include chemical pesticides, biological control, and the use of resistant varieties which contain *Dn* (*Diuraphis noxia*) resistance genes (Botha, 2021; Randolph et al., 2009; Weiland et al., 2008). However, new biotypes of RWA rapidly overcome host resistance in a gene-for-gene specific manner. To date, eight RWA biotypes have been described in the US, although two biotypes dominate (biotypes 1 and 2), and five biotypes have been found in South Africa (Botha, 2021). Seventeen *Dn* resistance genes have been identified, yet resistance breakdown remains a persistent issue (Li et al., 2018).

Plant Defense Against Aphids

Plant defenses against aphids fall into three main categories: antixenosis, which affects aphid colonization behavior; antibiosis, which negatively impacts aphid physiology, fecundity, and survival; and tolerance, which allows plants to sustain aphid infestations without significant yield loss (Nalam et al., 2019). Resistant cultivars may have a combination of these types of resistance mechanisms (Wang et al., 2004).

There are two primary models of aphid resistance in plants, specific resistance and general resistance, which follow principles established in plant-pathogen interactions (Smith & Boyko, 2007; Züst & Agrawal, 2016). In the specific resistance model, *R*-gene-mediated resistance is triggered by the recognition of introduced effectors. These effectors can originate from various sources in the aphid saliva including aphid proteins, bacterial proteins (derived from both endosymbiont and facultative microbes), as well as long non-coding RNA (Bos et al., 2010; Botha, 2021; Chaudhary et al., 2014; Chen et al., 2020; Elzinga & Jander, 2013; Nalam et al., 2019; Smith & Boyko, 2007; Yates & Michel, 2018). However, aphids can rapidly evolve to

evade *R*-gene detection through the emergence of new biotypes (Burd et al., 2006; Haley et al., 2004).

The general resistance model describes broad-spectrum plant defenses that vary between resistant and susceptible hosts. Unlike specific resistance, this model does not rely on *R*-gene recognition but instead leverages plant defense and hormonal pathways to mitigate aphid feeding damage. General resistance is primarily regulated by hormonal crosstalk between salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Morkunas et al., 2011; Smith & Boyko, 2007; Züst & Agrawal, 2016).

A key approach for identifying aphid-associated effectors involves proteomic analysis of aphid watery saliva. In multiple aphid species, this approach has revealed numerous candidate effectors, some of which have been functionally characterized (Atamian et al., 2013; Bos et al., 2010; Chaudhary et al., 2014; Elzinga et al., 2014; Mutti et al., 2008; Rao et al., 2013; Wang et al., 2015). For RWA specifically, early studies on the salivary proteome found that protein composition varied between biotypes and was distinctly different from other aphid species (Cooper et al., 2010; Nicholson et al., 2012).Nevertheless, no likely candidate effectors have been identified to date. Recently, Nicolis and colleagues (2022) generated a catalogue of 726 potential aphid effectors using whole-body transcriptomics, but none of these have been functionally validated.

Hormonal Regulation and Responses to Aphid feeding

Salicylic acid (SA) is primarily involved in defense against biotrophic pathogens and plays a key role in systemic acquired resistance (SAR), while jasmonic acid (JA) defense responses are

more effective against chewing herbivores and necrotrophic pathogens (Jones & Dangl, 2006). JA and ethylene (ET) can act synergistically to induce unique plant defenses (Dong et al., 2004) but JA is often antagonistic to SA signaling (Ding et al., 2016). Many insects, including aphids, manipulate these hormonal pathways to promote host susceptibility (Gao et al., 2007).

Aphid feeding modulates three major plant defense hormones: JA, SA, and ET (Thompson & Goggin, 2006). A "decoy" hypothesis was proposed that suggests that aphids induce SA while simultaneously downregulating JA-mediated defenses, effectively shifting plant immunity toward a defense strategy that is less detrimental to aphid success (Zhu-Salzman et al., 2005; Zhu-Salzman et al., 2004). Crosstalk between JA and SA can be mediated by WRKY transcription factors which influence a balance between these hormonal pathways (Kaloshian, 2004; Li et al., 2008; Smith & Boyko, 2007; Studham & MacIntosh, 2013; van Eck et al., 2010).

Many aphid-plant systems exhibit SA upregulation and activation of SA-mediated signals during resistant interactions with plants. These include plant interactions with greenbug (Zhu-Salzman et al., 2004), green peach aphid (Ellis et al., 2002; Moran & Thompson, 2001), and potato aphid (de Ilarduya et al., 2003). In these studies, JA is also upregulated for a short time, then is suppressed.

In RWA-infested plants, JA biosynthesis and signaling are significantly upregulated in resistant wheat interactions, with expression levels increasing up to 3.5-fold (Liu et al., 2011). Conversely, in susceptible interactions, JA-mediated defenses are often suppressed and instead, SA-related defenses are induced (Luna et al., 2018; Smith & Boyko, 2007; Smith & Clement, 2012). Most studies found that in both susceptible and resistant plants, SA is induced

during aphid infestation (Gao et al., 2007; Mohase & van der Westhuizen, 2002; Thompson & Goggin, 2006). However, (Mohase & van der Westhuizen, 2002) reported that SA was induced only in resistant interactions, suggesting context-dependent variation in SA signaling. In resistant barley plants, JA, ET, and auxin expression were elevated, suggesting a broader hormonal response to aphid feeding (Marimuthu & Smith, 2012).

In other aphid-plant interactions other aphid-associated factors, including honeydew or aphid endosymbionts, may contribute to hormonal modulation. For example, exogenous application of aphid honeydew from the pea aphid induced SA while simultaneously suppressing JA in broad bean plants (Schwartzberg & Tumlinson, 2014); the specific components in the honeydew that induce SA were not determined. GroEL a protein in *Buchnera aphidicola* from potato aphids (Chaudhary et al., 2014) triggered plant defense responses in *Arabidopsis* plants resulting in a negative impact for the aphid.

Aphid microbiomes and their roles in aphid-plant interactions

The rapid evolution of RWA biotypes is unlikely to be due solely to genetic changes in the aphid genome, as such plasticity would require widespread genomic rearrangements (Burger & Botha, 2017). Instead, the establishment of bacterial associations may contribute to RWA virulence, with microbial-derived effectors modulating plant defense responses. However, the role of microbes in aphid-plant interactions has not been well studied. Most studies on aphid-associated bacteria have focused on single bacterial species, largely overlooking the broader aphid microbiome's role in aphid aggressiveness and biotype evolution.

Nearly all aphid species harbor the primary bacterial symbiont *Buchnera aphidicola*, which provides essential amino acids that plant sap lacks (Buchner, 1965). Many aphid species also contain facultative (secondary) symbionts, which confer a range of benefits including nutritional supplementation, thermal tolerance and defense against predators (Montllor et al., 2002; Oliver et al., 2003; Su et al., 2015).

Studies have revealed distinct bacterial communities among aphid biotypes (Gauthier et al., 2015) and comparative studies of aphid microbiomes have identified common bacterial genera across species, including *Regiella*, *Serratia*, *Hamitonella*, *Pseudomonas*, *Acinetobacter*, *Pantoea*, *Enterobacter*, *Erwinia*, and *Staphyloccus* (Fakhour et al., 2018; Gauthier et al., 2015; Holt-Harris & Teague, 1916; Luna et al., 2018)

Despite these insights, the role of microbiomes in plant defense suppression and insect adaptability remains unclear. Further research is needed to determine if and how these microbial communities contribute which could inform novel strategies for aphid management by targeting symbionts or their effectors.

Research Objectives, Hypothesis and Scope of Dissertation

To address gaps in our knowledge of RWA-plant interactions and the possible roles of microbes in modulating those relationships, this dissertation aims to:

- Determine if microbes contribute to RWA aggressiveness or virulence on plants.
- Identify which microbes are involved and where they are located within the aphid.

- Investigate how these microbes influence plant defense responses.

We hypothesize that RWA-associated microbes promote aphid virulence by modulating plant hormone pathways to enhance aphid success.

This work is divided into three main chapters.

Chapter 2, Bacteria Associated with Russian Wheat Aphid (*Diuraphis noxia*) Enhance Aphid Virulence to Wheat, explores the RWA salivary and aphid microbiome composition. We found that RWA saliva is enriched in bacterial proteins. In addition, we characterized the microbiome of RWA. Our results indicate that RWA with high bacterial loads evade plant defenses more effectively than those with low titers of bacteria. Additionally, we demonstrated that microbes associated with RWA modulate plant hormones, in particular SA.

Chapter 3, *Erwinia iniecta* sp. nov., isolated from Russian wheat aphids, describes the identification and characterization *Erwinia iniecta* (renamed *Winslowiella iniecta* (Brady et al., 2022) as a novel aphid-associated bacterium. Comparative analysis of its genome with other *Erwinia* strains, including average nucleotide identity (ANI) analysis, confirmed its classification as a new bacterial species. Furthermore, we examined its genome for potential virulence mechanisms that may influence plant-aphid interactions.

Chapter 4, Bacteria associated with Russian wheat aphid honeydew modulate defense response gene pathways in barley, investigates the transcriptomic response of aphid-associated bacteria and aphid-honeydew associated bacteria, with a focus on their role in plant defense modulation. We demonstrate that plant defense response patterns during aphid feeding are similar to those in honeydew and in bacterial treated plants. We highlight changes in plant

hormone pathways and their regulation that are activated in microbe-enriched honeydew and RWA, and postulate how these changes are involved in aphid success.

Together, these findings provide valuable insights into wheat and barley phytobiomes and highlight the importance of microbiomes in aphid-plant interactions. Understanding how bacterial communities influence plant defenses may help inform pest management strategies by identifying novel targets for plant resistance. In an era where the economic burden of pest control significantly influences agricultural decisions, and with global temperatures rising – potentially expanding the geographic range of RWA infestations– identifying new resistance strategies is increasingly critical.

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CHAPTER 2: BACTERIA ASSOCIATED WITH RUSSIAN WHEAT APHID (*DIURAPHIS NOXIA*) ENHANCE APHID VIRULENCE TO WHEAT[1]

2.1 OVERVIEW

Phenotypic responses to biotic stresses are often studied as the interactions between two species; however, in the phytobiome, these responses frequently result from complex interactions involving several organisms. Here, we show that variation in chlorosis caused by Russian wheat aphid (*Diuraphis noxia*) feeding is determined, in part, by aphid-associated bacteria. Proteomic analysis of fluids injected into a sterile medium by the aphid during feeding indicate that 99% of the proteins are of bacterial origin. Of these, the greatest proportion are produced by bacteria in the family *Enterobacteriaceae*. Bacteria from five genera in three families that have the capacity to produce these proteins were isolated directly from aphids as well as from wheat leaves only after *D. noxia* feeding. By themselves or in combination, these bacteria were not virulent to wheat, even at high inoculum levels. Metagenomic analysis showed that the same five *D. noxia*-associated genera dominated the non-*Buchnera* component of the aphid microbiome, and that representation of these genera was reduced in aphids from colonies established after isolation of newborn nymphs from their mothers prior to feeding ('isolated' aphids). Isolation or treatment with antibiotics reduced bacterial numbers, and these aphids caused less feeding damage on wheat than non-isolated or non-antibiotic treated aphids. Our data show that bacterial proteins are a significant component of Russian

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wheat aphid saliva, that the bacteria producing these proteins are associated with aphids and plants fed upon by aphids, and that these aphid-associated bacteria facilitate aphid virulence to wheat.

2.2 INTRODUCTION

Aphids have intimate associations with various bacteria. These associations range from mutualistic to pathogenic (Buchner, 1965) and are classified as either obligate or facultative (Gil et al., 2004). The obligate endosymbiont *Buchnera aphidicola* is essential for aphids because it synthesizes several amino-acids that plant sap lacks (Douglas & Prosser, 1992), an association which was established over 180 Mya (Moran et al., 1993). Other non-obligatory secondary endosymbionts, such as "*Candidatus* Hamiltonella defensa", "*Ca.* Serratia symbiotica", "*Ca.* Regiella insecticola", and *Rickettsia* spp. are not required for aphid survival but enable aphids to defend themselves against natural enemies (parasitoids, fungi or bacteria) or promote high temperature tolerance (Montllor et al., 2002; Oliver et al., 2003; Scarborough et al., 2005). Besides endosymbiotic bacteria, aphids carry other bacteria that have not received as much attention. Facultative bacteria such as *Erwinia* spp., *Pantoea* spp., *Dickeya dadantii,* and *Pseudomonas syringae* are carried by, or may be ingested by aphids, and may replicate within the aphids (Campillo et al., 2015; Correa et al., 2012; Costechareyre et al., 2012; Harada et al., 1997; Stavrinides et al., 2010). In contrast to obligate symbionts that are transmitted vertically (Douglas, 1998), these associated bacteria are usually horizontally transmitted (Oliver et al., 2010; Oliver et al., 2012). In recent years, several studies have characterized all of the bacteria present in aphid samples by deep sequencing of 16S rRNA gene sequences (Bansal et al., 2014;

Gauthier et al., 2015; Gil et al., 2004; Jing et al., 2014; Jones et al., 2011; Jousselin et al., 2016). A common theme throughout these studies is the reduced presence of facultative bacteria relative to the primary and secondary endosymbionts.

Aphids are piercing-sucking insects that use a stylet to probe intercellularly through the mesophyll until reaching the phloem sap, where feeding begins (Miles, 1999). During probing and feeding, aphids secrete saliva into the plant, and the saliva is thought to suppress plant defense responses (Miles, 1999). Many studies have characterized the salivary proteome of aphids in search of candidate virulence proteins (effectors) (Bos et al., 2010; Carolan et al., 2011; Cooper et al., 2010; Harmel et al., 2008; Mugford et al., 2016; Nicholson et al., 2012; Nicholson & Puterka, 2014; Vandermoten et al., 2014). Several aphid proteins were proposed to be effectors from these studies, but only one of these aphid proteins was shown to be an effector (Mugford et al., 2016). Evidence is lacking for effectors in other aphid species, notably the agricultural pest Russian wheat aphid, *Diuraphis noxia* (Kurdjumov) (Hemiptera: Aphididae). Although a few studies have characterized the salivary proteome of *D. noxia*, no study to date identified candidate aphid-produced virulence proteins (Cooper et al., 2010; Nicholson et al., 2012). Similarly, genome characterizations of *D. noxia* revealed no candidate effectors (Burger & Botha, 2017; Nicholson et al., 2015).

Russian wheat aphid is particularly damaging to wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare L.*). Symptoms of aphid feeding include leaf chlorosis, plant stunting, leaf rolling, and plant desiccation, and these ultimately lead to yield reduction (Burger & Botha, 2017; Webster et al., 1987; Zwer et al., 1994). Resistant cultivars are employed for aphid

management, however, aphids quickly overcome plant resistance by evolving new biotypes. Assignment of these biotypes is based on differential virulence to wheat (i.e., *D. noxia* biotype 1 is less virulent than biotype 2) (Nicholson et al., 2012).

The abundance, diversity, and role of facultative bacteria colonizing aphid species is still relatively unknown and likewise, attempts at characterizing the aphid-associated bacterial composition of *D. noxia* are scant (Anathakrishnan et al., 2014; Campillo et al., 2015). In one study, a gut transcriptomic analysis for *D. noxia* found higher bacterial sequence diversity in Russian wheat aphid biotype 2 relative to biotype 1 (Anathakrishnan et al., 2014). In our previous study, we isolated several bacterial genera and identified the aphid-associated bacterium *Erwinia iniecta* (Campillo et al., 2015). Other than these studies, no information is available on facultative bacteria associated with Russian wheat aphid or their role in aphid feeding and establishment.

In this study, we characterized the proteome of *D. noxia* saliva and showed that few aphid salivary proteins were present, but instead, many proteins of bacterial origin, predominantly from three genera in the *Enterobacteriaceae*, were detected. Bacteria from these same genera were isolated from artificial diets and from wheat plants, but only those that were fed upon by *D. noxia*. We show that *D. noxia* with reduced bacterial populations cause less chlorosis in wheat, and that the microbiomes of these bacterial-deficient aphids are underrepresented in many of the same bacterial genera detected in aphid saliva. Based on these findings, we hypothesize that *D. noxia* delivers bacteria to the plant during feeding and that these bacteria enhance aphid virulence.

2.3 MATERIALS AND METHODS

Plant growth and aphid rearing. Parthenogenic colonies of *D. noxia* biotypes 1 and 2, originally collected from field infestations of wheat in CO, were reared and maintained on susceptible wheat plants (*Triticum aestivum* L.) cultivars 'TAM 107', 'Yuma' and 'Byrd' following the methods described in Randolph et al. (2009). Wheat plants susceptible to both aphid biotypes 1 and 2, were grown in a greenhouse with 16 h light/8 h dark at 23 ± 3 °C, in Promix soil (Premier Horticulture, Dorval, QC, Canada). The infested plants were held in 60 × 60 × 60 cm insect cages (Bug Dorm Store, Megaview Inc., Taichung, Taiwan) to avoid contamination with non-experimental aphid species.

Aphid saliva collection. Russian wheat aphid biotypes 1 and 2 were reared on susceptible wheat cultivar 'TAM 107' for several generations prior to the start of the experiment. Saliva collection plates were constructed per Cooper et al. (2010). Briefly, the plates were constructed under sterile conditions by stretching a thin membrane of Parafilm (Pechiney Plastic Packaging, Chicago, IL) over an inverted 60 × 15 mm Petri dish. Before sealing with Parafilm, 1.2 ml of sterile sucrose solution (15%, pH 7.2) was added, forming a feeding sachet. A 50 mm O-ring was placed on top of the Parafilm and ~500 apterous adult aphids were then placed on each plate. Aphids were incubated at 22 °C under a yellow light at a 14 h light/10 h dark photoperiod as described (Cooper et al., 2010). The entire experiment was replicated three times with 130 cages constructed per biotype (130 cages × 2 biotypes × 3 replicates = 780 cages total).

Collection plates incubated without aphids served as controls. After 48 h of feeding, aphids were removed with a paintbrush, and the surface of the feeding sachet was rinsed with sterile

distilled water. For protein analysis, the diet was collected by making an incision in the Parafilm at the edge of the dish, rinsing the underside of the Parafilm by pipetting the diet up and down a few times to ensure the collection of any adhering proteins, and finally removing the diet with the pipette. This entire sample was collected into 50 ml centrifuge tubes and the proteins were precipitated from the artificial diet by the addition of 10% trichloroacetic acid, 60% acetone, 1% *β*-mercaptoethanol (final concentrations) and incubating the samples at –20 °C overnight. Precipitated proteins were collected by centrifuging at 5,000 *g* for 30 min at 4 °C and removing the supernatant. The walls of each 50 ml tube were washed extensively with ice-cold acetone to remove residual sucrose and the sample was pelleted by centrifugation at 7,000 *g* for 10 min at 4 °C. Samples were dried using a vacuum concentrator prior to solubilization in the appropriate solutions for downstream analysis.

iTRAQ Analysis. Three biological replicates were analyzed for each of the two aphid biotypes. Each biological replicate was comprised of pools of saliva collected from thousands of individual insects from three clonal lineages of each *D. noxia* biotype. Protein pellets were solubilized in 0.1% ProteaseMax (PMAX) surfactant (Promega), 50 mM triethylammonium bicarbonate (TEAB), and total protein concentrations were estimated using a Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA). The integrity of the protein samples was assessed by separation and visualization in 10% SDS-gels as described (Ramsey et al., 2015). Samples were each aliquoted at 1 µg μl^{-1} starting protein in a total volume of 65 µl.

For reduction and cysteine blocking steps, 50 mM TEAB and 0.1% PMAX was added to the solution. Samples were reduced with 5 mM tris-(2-carboxyethyl)-phosphine (TCEP, pH 8.5) for

20 min at 55 °C, and 10 mM methyl-methane-thiosulfonate (MMTS) was added to each sample and incubated for 20 min at room temperature. A methanol/chloroform precipitation was carried out at 4 °C for 75 min after adding the following to each sample: 4× volume methanol; 1× volume chloroform; 3× volume deionized water. After centrifugation (15,000 *g* for 5 min at 4 °C) and removal of the supernatant, methanol was added to each pellet at 3× the sample volume, and the pellets were gently vortexed followed by centrifugation at 4 °C for 5 min. The methanol was removed and the pellet was air dried briefly. The pellets were solubilized in 0.1% PMAX/50 mM TEAB. Sequence grade modified trypsin (1µg/sample) was added and samples were incubated overnight at 37 °C. The digested samples were subjected to vacuum centrifugation until almost dry, and then 30 µl of 500 mM TEAB was added to each. The iTRAQ labeling reagents (Applied Biosystems, Foster City, CA) were each reconstituted in 50 µl of isopropanol and added to each sample as directed by the manufacturer. The samples were incubated at room temperature for 2 h during the labeling reaction. TFA was added to each sample to bring the pH below 4. All six biological replicates labeled with individual iTRAQ reporter mass tags were then pooled to one single tube and mixed for analysis.

Combined, iTRAQ-labeled samples were analyzed using two-dimensional liquid chromatography MudPIT(Washburn, 2004), using a two-dimensional vented column setup with a Proxeon nano-flow high-performance liquid pump (Taylor et al., 2009). A total of 50 µg of sample was loaded onto a tri-phasic, fused silica capillary column: 250 µm internal diameter packed with 3 cm of 5 µm Aqua C18, followed by 3 cm of 5 µm Luna SCX and 2 cm of 5 µm Aqua C18. A total of seven salt steps were used. For each salt step, peptides were eluted on an analytical column of a 100 µm internal diameter capillary with a 5 µm pulled tip and packed

with 15 cm of 3 µm Aqua C18 on line with an LTQ Orbitrap Velos (Thermo Fisher) as described in (Scuoppo et al., 2012). Data were acquired in profile mode using the following parameters: for full-scan Fourier transform mass spectrometry, resolution = 60,000, m/z = 380–1,700 and the 10 most intense ions were fragmented with higher-collision dissociation at a normalized collision energy of 40% and an activation time of 0.1. Minimum threshold signal was at 5,000 and isolation width at 1.2. Dynamic exclusion settings were repeat count 1, repeat duration of 30, exclusion list size 500, exclusion duration 60 and exclusion mass width 10 p.p.m. All mass spectrometry data files associated with this experiment can be found on ProteomeXChange, (Project accession PXD010663).

Protein identification. The MS and MS/MS data collected were submitted to Mascot v.2.3 (Matrix Science, Boston, MA) using an in-house MASCOT server for database interrogation. The data were searched against the entire NCBI non-redundant database, which contained the *A. pisum* gene models (download date: January 2011). This initial search revealed that the samples were comprised predominantly of plant pathogenic bacterial proteins. Therefore, we also searched the data using a custom database that contained a combination of predicted proteins from *A. pisum* (from NCBI, download date 7-23-2014), sequences from common contaminant proteins, as well as prokaryotic proteins predicted from microbes known to be associated with aphids, including *Erwinia* spp., *Pantoea* spp., *Enterobacter* spp., *Acinetobacter* spp. and insect endosymbionts belonging either to the *Enterobacteriaceae* (*Photorhabdus* spp., *Candidatus* Hamiltonella defensa, *Candidatus* Regiella insecticola, *Arsenophonus* spp., *B. aphidicola*, *Candidatus* Carsonella ruddii and *Serratia symbiotica*) and the *Rickettsieae* (*Wolbachia* spp. and *Rickettsia* spp.). The latter custom database had a total of 2,033,158 sequences. The following

search parameters were used: methylthio-cysteine as a fixed modification, methionine oxidation as a variable modification, and one missed tryptic cleavage. The searches were done with a mass error tolerance of 25 ppm for precursor ions and 0.1 Da for fragment ions. As described in the Scaffold Q+ (version Scaffold_4.4.1.1, Proteome Software Inc., Portland, OR) Publication Report, iTRAQ peptide and protein identifications were quantified as follows. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold local false discovery rate algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Channels were corrected according to the algorithm described in i-Tracker (Shadforth et al., 2005) as follows: Acquired intensities in the experiment were globally normalized across all acquisition runs. Individual quantitative samples were normalized within each acquisition run. Intensities for each peptide identification were normalized within the assigned protein. The reference channels were normalized to produce a 1:1fold change. All normalization calculations were performed using medians to multiplicatively normalize data.

Bacterial isolation and characterization. Bacteria were isolated from *D. noxia* biotype 1 and 2 bodies and artificial diets as previously described (Campillo et al., 2015). Sterile artificial diets handled in the same way, but not exposed to aphids, served as a control. To isolate bacteria from plant material, 24 aphids were placed on 14-day-old wheat leaves. After 2 weeks,

the aphids were gently removed from the leaf surface with a paintbrush and leaf samples were taken from the symptomatic portion of five different leaves. Samples were treated with 10% bleach for 10 s, rinsed with sterile water, and then ground in 100 µl of distilled water using a sterile glass rod. Leaves that had not been exposed to aphids served as controls. Isolation experiments were performed twice, with four replications per experiment. Suspensions from all of the above sources were plated on Eosin Methylene Blue agar (Holt-Harris & Teague, 1916) or nutrient agar medium and incubated 72 h at 28°C. Single colonies were selected and streaked onto a new agar plate. This step was performed at least three times to obtain pure bacterial cultures.

To determine the *rrs* gene (encoding 16S small ribosomal subunit) sequence for phylogenetic purposes, bacterial genomic DNA was extracted using an Easy DNA kit (Invitrogen, CA) per the manufacturer's instructions. Gene amplifications were performed by polymerase chain reactions (PCR) containing 5× PCR Phusion HF buffer (Finnzymes, Finland), 0.2 mM dNTP, 0.5 mM forward (FD1, 5'-agagtttgatcctggctcag-3') and reverse (RP2, 5'-acggctaccttgttacgactt-3') primers (Weisburg et al., 1991), 25 ng extracted genomic DNA, 3% DMSO and 0.5 unit of Phusion Taq polymerase (Finnzymes, Finland) in 20 µl aqueous solution. An initial denaturation step was performed at 98°C for 1 min followed by 35 cycles: 10 s at 98°C, 30 s at the annealing primer temperature and 1 min at 72°C; the reaction ended with a final extension 4 min at 72°C. Amplicons were sequenced in both directions by the Colorado State University Proteomics and Metabolomics Facility (Fort Collins, CO) after a purification step using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin). Sequence analysis and phylogenetic tree generation were performed using the software MEGA version 5 (Tamura et al., 2011).

Bacterial multiplication in sucrose rich media. Ability of aphid-associated bacteria to multiply in sucrose rich media was measured in two ways. First, we cultured several aphid-associated bacteria (*Acinetobacter* sp. B114, *Arthrobacter* sp. B117, *E. iniecta* B120, *Pantoea* sp. B151, and *Enterobacter* sp. B156) in media with or without a high sucrose content. The pH for all media was adjusted to 7.0. Media included: Luria-Bertani broth (LB), LB with 15% sucrose, sterile distilled water, sterile distilled water with 15% sucrose. For the experiment, three cultures of each bacteria were grown in an overnight liquid culture of nutrient broth for 16 h, washed two times, resuspended in distilled water, and adjusted to an OD_{600} of 0.2. After 10-fold serial dilutions, 200 µl aliquots of three technical replicates of each of the bacterial cultures were pipetted into a 96 well microplate. The plates were incubated at 28 °C with agitation in a plate reader (PowerWave HT, BioTek, U.S.), and the OD_{600} was measured hourly for 72 h. In a second approach, we determined multiplication of bacteria in sucrose-rich artificial diets fed on by aphids by measuring turbidity (OD_{600nm}) of sterile 15% sucrose solution and 15% sucrose solution from artificial diets after 24 and 48 h of aphid feeding.

Contribution of *D.* noxia-associated bacteria to aphid virulence. The contribution of bacteria to *D. noxia* virulence was assessed after reducing titers of bacteria associated with aphids in two ways. In these studies, the more virulent biotype, *D. noxia* biotype 2 was used. First, we fed aphids an artificial diet supplemented with antibiotics. Preliminary experiments showed that an artificial diet supplemented with 50 µg/ml rifampicin and 50 µg/ml chlortetracycline for 48 h maximized aphid survival while minimizing bacterial populations. The virulence of the antibiotic treated aphids was compared with aphids fed an artificial diet without antibiotics for 48 h. Four 7-day-old TAM 107 wheat plants were infested with 50

antibiotic-treated or 50 untreated aphids per plant. This experiment was repeated four times for a total of 16 plants per treatment. Seven to ten days after infestation, the total number of aphids were counted (to measure any changes due to births or deaths) and the proportion of total leaf area that was chlorotic was measured using Image J 1.47v (Schneider et al. 2012). To assess any possible effects of antibiotics on the reproductive ability or survivorship of the aphids, we used a t-test to compare the number of antibiotic-treated and untreated aphids after two weeks on wheat plants. We used an ANCOVA to assess the effects of the antibiotic treatment on the logit-transformed proportion of chlorotic leaf tissue that developed after adjusting for the number of aphids per leaf (the covariate) (Lindman, 2012).

To avoid potential effects of antibiotics on the obligate aphid symbiont *B. aphidicola* (Koga et al., 2003; Koga et al., 2007), we used a second method to reduce titers of facultative bacteria. Prior to each experiment, we established colonies of aphids on uninfested wheat plants from individual nymphs that had been isolated from their mother at birth before they initiated feeding. This technique allowed us to establish colonies of 'isolated' aphids free of facultative bacteria that are transmitted from mother to offspring during co-feeding. Control aphid colonies (‘co-fed’ aphids) were established by allowing the new-born aphid to co-feed with its mother and siblings as the colony established. Twenty-four 14-day-old TAM 107 plants were infested with 15 adult aphids from either colony (12 plants with ‘isolated’ aphids, 12 plants with ‘co-fed’ aphids). After 10 days, aphid numbers were recorded and the proportion of total leaf area that was chlorotic was measured using Image J 1.47v (Schneider et al., 2012). Bacterial colony counts from randomly sampled aphids were conducted at the end of the experiment to ensure these aphids were still free of horizontally transmitted bacteria. Ten adult

aphids from each plant were pooled and crushed into 50 µl of Carlson's Solution and then the liquid was transferred onto nutrient agar amended with 50 mg/l cycloheximide (to eliminate fungal growth) to measure bacterial numbers. The number of bacteria isolated from 'isolated' aphids and 'co-fed' aphids were log (N+1) transformed and compared with a t-test. We used an ANCOVA to examine the effect of 'isolated' vs. 'co-fed' aphids on the proportion of chlorotic leaf tissue induced (logit-transformed) after adjusting for the effects of the number of aphids present (the covariate).

Sample collection, DNA extraction and sequencing of 16S rRNA amplicons. Ten samples containing either ten 'isolated' or ten 'co-fed' adult aphids (biotype 2, five samples per treatment) were subjected to 16S rRNA amplicon sequencing. An additional sample was sequenced that contained a mock community of 12 known bacterial isolates in equal concentrations (Supplementary Table S2.1) to assess contamination and sequencing errors. All aphids were reared on 'Yuma' wheat plants for approximately 12 generations before collection for DNA extraction. Under sterile conditions, aphid DNA was extracted using the MoBio PowerSoil extraction kit as directed by the manufacturer, with the following deviations: (i) in the PowerBead Tubes, aphid samples were vortexed in a FastPrep bead beater at a speed of 5.0 for 2 min, (ii) after the initial vortex step, 20 µl of proteinase K (20 mg/ml) was added to each sample and vortexed briefly, and (iii) samples were then incubated at 60°C for 1 h. The protocol was followed until the elution step, where 30 µl of solution C6 was added (first elution), and a second elution was performed with 20 µl of solution C6. In addition to the aphid samples, two tubes with no aphids served as negative controls to ensure reagents and technique did not contribute to contamination. For quality control, PCR using theV4 region of the 16S rRNA

subunit with the 515F/806R primer set was conducted at Colorado State University; all DNA from aphid samples amplified, but samples with no aphids did not amplify (Supplementary Fig. S2.1), suggesting contamination had not occurred, and were therefore not further processed.

Bacterial strains used for the mock community (positive control, shown in Supplementary Table S2.1) were grown overnight at 28°C or 37°C on nutrient agar (Becton, Dickinson and Company, Franklin Lakes, New Jersey). Genomic DNA was prepared using the EasyDNA kit (Life Technologies, Grand Island, NY) according to manufacturer's recommendations, except the final product was recovered in 50 µl sterile molecular grade water. After DNA extraction and quantification, equal amounts of DNA from each strain were added together to create the mock community.

The first elutions of the aphid samples and the mock community were used for PCR amplification, pooling, library preparation and paired-end sequencing (MiSeq; 2x150 bp pairedend reads) using V4 region of the 16S rRNA gene with the 515F/806R primers at SeqMatic (Freemont, CA).

16S rRNA data processing and analyses. Sequencing reads were demultiplexed, and forward reads were processed using the open-source bioinformatics platform Qiime 2 version 2017.10 (Caporaso et al., 2010). Briefly, sequence variances were detected using the DADA2 pipeline (Callahan et al., 2016) where chimeric sequences and phiX reads were removed. Taxonomy was assigned using a pre-trained Naïve Bayes classifier (Bokulich et al., 2018) that was trained on the Greengenes database, but was also hand curated by alignment of sequences of unknown taxonomy from the feature table to genomes of *D. noxia*-associated bacteria. Only

sequences that matched nucleotides of sequenced genomes 100% were added to the feature table (Supplementary Fig. S2.2). Phylogenetic trees were generated by aligning sequences using the mafft program (Katoh & Standley, 2013), and then alignments were filtered to remove variable positions. Once alignments were filtered, FastTree (Price et al., 2010) was used to create a phylogenetic tree with midpoint rooting. As the focus of this study was to look at facultative bacteria, sequences that mapped to *Buchnera* were removed and the remaining reads were rarified to a sampling depth of 425 reads. Alpha diversity was measured by observed operational taxonomic units (OTUs) and Faith's phylogenetic diversity (Faith, 1992). Beta-diversity was evaluated using unweighted unifrac distances which were used to construct a Principal Component Analysis to further investigate treatment differences. A PERMANOVA statistical test was used to determine if the two treatment groups differed at the beta diversity level. We tested the relative abundance of each taxon in the two aphid groups using the analysis of composition of microbiomes (ANCOM) compared the relative abundance of sequence variants between the two aphid treatments.

Inoculation of wheat leaves with bacteria Approximately 5 µl of bacterial resuspensions (1 x 10^8 CFU ml^{-1}) were infiltrated into three-week-old 'Byrd' wheat leaves using a needle-less syringe (Reimers & Leach, 1991). One to two representative aphid-associated bacterial isolates from each genus were used for inoculations, including: *Acinetobacter* spp. 114; *Erwinia* sp. 116 and 120; *Arthrobacter* sp.117; *Pantoea* sp. 151; and *Enterobacter* sp. 156. Bacteria were infiltrated into the adaxial surface of plants individually or in combination (equal numbers of each bacterial strain). Controls for the experiment included a water control and *Xanthomonas*

translucens (strain UPB787), a known pathogen of wheat (Langlois et al., 2017). Plants were observed at 24, 48 and 72 h for signs of chlorosis, browning, or water-soaking.

Microarray data analysis. Expression of genes involved in the jasmonic acid (JA) and salicylic acid (SA) biosynthetic pathways in wheat were determined by re-analysis of data from a previously published microarray analysis (Botha et al., 2010). The original experimental design included RNA extracted from a susceptible wheat cultivar (Gamtoos) infested with Russian wheat aphid biotype 1 for 0, 5, and 48 h, and included three replicates per treatment. Probe set IDs, annotated with respect to the JA or SA biosynthetic pathways, were extracted from the full data set and $\log_2$-fold changes in expression relative to the uninfested 0 h time point were calculated for each probe set ID at 5 hours post infestation (hpi) and 48 hpi. Probe sets with an absolute value of $\log_2$-fold change > 1 were considered to be differentially expressed, and probe sets with differential expression at either 5 or 48 hpi were included in our analysis.

Phytohormone quantification. JA and SA accumulation was assessed after aphid feeding on wheat leaves. Fourteen-day-old wheat plants (Yuma) were heavily infested with approximately 0.5 g of aphids (≈ 2500 aphids) which were either 'isolated' aphids or 'co-fed' aphids, or plants were left uninfested. After 6, 24, or 48 h, aphids were gently brushed off leaves using a fine bristled paint brush and the leaves were quickly weighed and placed in liquid nitrogen and stored at −80ºC. Each time point consisted of three replicates and within each replicate, leaves of the same age from two or three plants were sampled and pooled to meet the required sample weight of 100 mg of fresh tissue. Frozen leaves were ground using a tissue lyser (TissueLyser, Retsch, Qiagen), extracted with methanol (Almeida Trapp et al., 2014), dried

under a stream of nitrogen, and shipped to The Samuel Roberts Noble Foundation (Ardmore, OK). Samples were re-suspended in 100% methanol containing internal standards for JA and SA prior to separation, detection, and quantification (Pan et al., 2010; Watson et al., 2015). Relative amounts of SA and JA were based on comparisons to the labeled hormones. A two-way ANOVA was performed to evaluate the effects of time point (6, 24, or 48 hpi) and aphid treatment (isolated aphids, co-fed aphids, or no aphids). Hormone measurements were log transformed to meet assumptions of normality and analyzed using JMP Pro version 12.0.1. Differences between treatment levels were determined using a Tukey's HSD.

2. 4 RESULTS

***Diuraphis noxia* saliva contains mostly bacterial peptides.** To identify proteins secreted by aphids during feeding, a whole proteomic analysis of artificial diets fed on by *D. noxia* biotypes 1 and 2 were collected and analyzed using Mud-PIT and iTRAQ analyses. No proteins were detected in control artificial diets (which were not fed upon by *D. noxia*) after separation in SDS-denaturing gels or by Bradford protein assays (data not shown).

A total of 1,734 proteins were identified when the data were searched against all proteins in the NCBI database. Only thirteen insect proteins were identified, and these were only from the Aphididae. These proteins included apolipophorin, glucose dehydrogenase, trehalase and alanyl aminopeptidase, and S-adenosyl-L-homocysteine hydrolase (Supplementary Table S2.2), and all were consistently found in both biotypes 1 and 2 at a similar abundance, with the exception of S-adenosyl-L-homocysteine hydrolase, which was detected only in biotype 2.

Strikingly, of the 1,734 identified, most proteins (78%) matched with bacterial proteins, particularly with those found in the *Enterobacteriaceae* (57%) (Fig. 2.1A). Proteins matching four genera, *Erwinia*, *Pantoea*, *Enterobacter* and *Acinetobacter*, were especially dominant. This observation was true for all three replicates of both *D. noxia* biotypes 1 and 2.

To expedite database searching and to eliminate redundancy found in the large NCBI database, we constructed an in-house database that, guided by the NCBI search results, contained a combination of predicted proteins from aphids and prokaryotes. The prokaryotic proteins were predicted from microbes known to be associated with aphids, including *Erwinia* spp., *Pantoea* spp., *Enterobacter* spp., *Acinetobacter* spp. and insect endosymbionts belonging to the *Enterobacteriaceae* (*Photorhabdus* spp., *Ca.* Hamiltonella defensa, *Ca.* Regiella insecticola, *Arsenophonus* spp., *B. aphidicola*, *Ca.* Carsonella ruddii and *Serratia symbiotica*) and the *Rickettsieae* (*Wolbachia* spp. and *Rickettsia* spp.). Comparisons of the *D. noxia* salivary proteins to this database revealed a total of 1,162 proteins comprising 562 protein families with more than 95% probability of identification with a minimum of two matching peptides (see Luna et al. 2018). Among them, proteins from four genera *Erwinia*, *Pantoea*, *Enterobacter,* and *Acinetobacter* were predominant, representing 52%, 23%, 9.5%, and 10% of the total matches, respectively (Fig. 2.1B). Proteins predicted to be from the insect endosymbionts *Photorhabdus* spp. *Arsenophonus* spp., *Ca.* Regiella insecticola, *Ca.* Hamiltonella defensa and *Serratia symbiotica* together totaled only 4.1%. Consistent with comparisons to the entire NCBI non-redundant database (Fig. 2.1A), only 1% of the proteins detected were of aphid origin. No matches were observed with other possible aphid endosymbionts, such as *Ca.* Carsonella ruddi, *B. aphidicola* or any members of the *Rickettsieae*.

The bacterial proteins identified in the artificial diet after feeding were all associated with bacterial housekeeping functions (Supplementary Fig. S2.3). Most were associated with metabolism (37%), ribosome and protein synthesis (16%), DNA maintenance and gene expression (13%), membrane transporters (7.8%), membrane synthesis and maintenance (8%), proteases and chaperones (5.7%), and stress (5.5%). Three bacterial virulence factors associated with virulence-related outer membrane proteins and the type VI secretion system were identified (type VI secretion ATPase, ClpV1 family; VI secretion system effector, Hcp1 family; type VI secretion system effector, Hcp family).

As sucrose is an important carbon source for many bacteria, we conducted an experiment to determine if high number of bacterial peptides in artificial diets fed on by *D. noxia* were an artifact of an environment conducive to bacterial growth. Aphid-associated bacteria do not multiply in the sucrose rich artificial diets (Supplementary Fig. S2.4) nor did turbidity (OD_{600nm}) change of artificial diets after 48 h of aphid feeding (Supplementary Table S2.3).

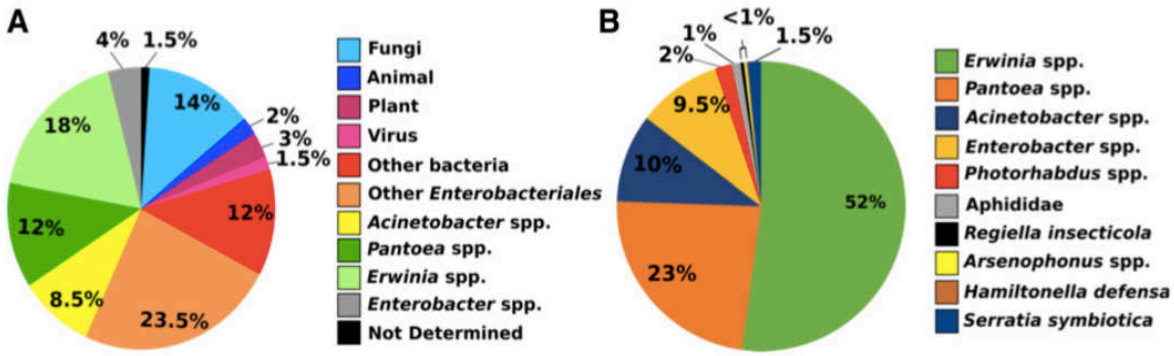

Fig. 2.1. The *Diuraphis noxia* salivary proteome is mostly composed of bacterial proteins, with very few insect proteins detected. A. Analysis of *D. noxia* saliva by MASCOT comparison against the entire NCBI non-redundant database. B. Comparison of *D. noxia* salivary proteins against a custom-built database that includes protein sequences from the Aphididae, and from prokaryotes known to be associated with aphids.

***Diuraphis noxia* release bacteria during feeding.** We previously demonstrated that bacteria can be cultured from artificial diets fed upon by *D. noxia* (Campillo et al., 2015). Based on our proteome study, *D. noxia* was expected to release Gram-negative bacteria, predominantly in the family *Enterobacteriaceae*. Given that *D.* noxia biotype 2 is the more virulent aphid and that there was no difference in overall bacterial composition in the proteome study between the two biotypes, biotype 2 was chosen for this study. To determine if the proteome predictions corresponded with culturable bacteria, taxonomic groupings were determined for isolates from three sources: (i) artificial diets, (ii) *D. noxia* and (iii) wheat leaves. Bacteria were not recovered from artificial diets or surface sterilized wheat leaves that had not been exposed to aphids.

Phylogenetic analysis based on the *rrs* (16s rRNA) sequences placed the isolates into three bacterial families: *Paenibacillaceae*, *Moraxellaceae*, and *Enterobacteriaceae*. Twelve *Paenibacillaceae* strains, isolated from crushed aphids or wheat leaves after aphid feeding, exhibited more than 99% DNA sequence identity, and all clustered into the genus *Paenibacillus* (data not shown). Since the *Paenibacillus* were not isolated from artificial diets after *D. noxia* feeding, they were not included in further phylogenetic analyses.

Twenty-three *Enterobacteriaceae* and *Moraxellaceae* isolates (Supplementary Table S2.4), which were recovered from the crushed aphids and from artificial diets and wheat leaves probed by aphids, grouped into five different clusters at the genus level (Fig. 2.2). These five clusters show more than 99% identity with five genera: four within the *Enterobacteriaceae* (*Erwinia*, *Pantoea*, *Enterobacter*, and *Arthrobacter*) and one in the Moraxellaceae

(*Acinetobacter*). One group of *Erwinia* isolates that was frequently recovered from all three sources was recently named *Erwinia iniecta* (Campillo et al., 2015). These results are consistent with the results of our proteome analysis. Furthermore, the same groups of bacteria were recovered from bodies of both biotypes of *D. noxia*, as well as from artificial diets and surface sterilized wheat leaves that had been in contact with or infested with *D. noxia*. Thus, we propose that *D. noxia* delivers bacteria, and predominantly *Enterobacteriaceae*, into plants via the stylet during feeding.

Aphid-associated bacteria enhance *D. noxia* virulence. To facilitate studies to determine the role of aphid-associated bacteria in virulence to plants, we established colonies of aphids with reduced bacterial numbers in two ways. First, we generated aphid populations with reduced bacterial numbers by treating them with antibiotics (rifampicin and chlortetracycline) for 48 h. Antibiotic treatment led to a reduction of bacterial numbers by 10-100 fold in comparison to untreated controls (data not shown). Plants fed upon by aphids not treated with antibiotics exhibited more chlorotic symptoms than plants fed upon by aphids after antibiotic treatment (partial-$F_{1,28}$ = 10.94, P = 0.0026; Supplementary Fig. S2.5A), indicating a role for the bacterial communities in RWA virulence. Antibiotic treatment did not negatively affect aphid survival or reproduction, as seen by the similar numbers of aphids recovered from susceptible wheat plants 7-10 days after feeding with antibiotic-treated or untreated aphids (t_{21} = 0.88, P = 0.39; Supplementary Fig. S2.5B).

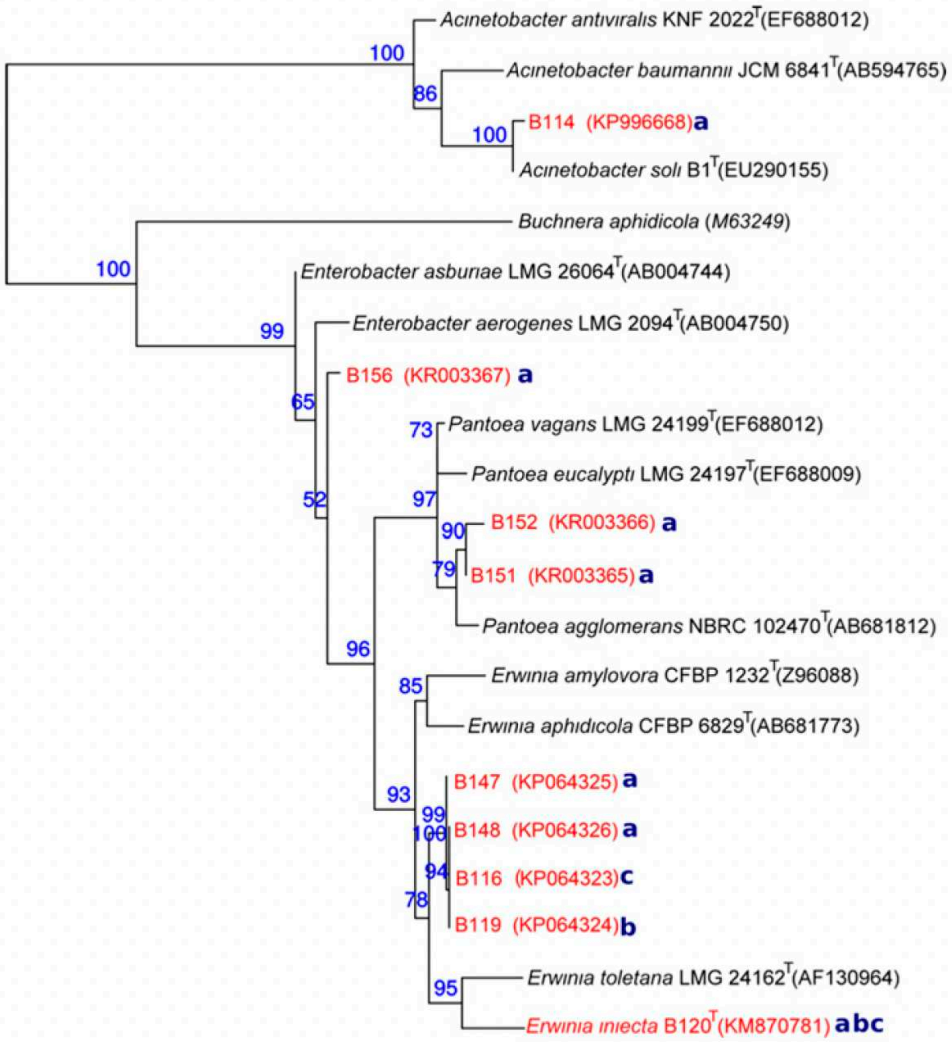

Fig. 2.2. Neighbor joining tree based on 16S rDNA (*rrs*) gene sequence alignment of bacteria isolated from artificial media fed on by *D. noxia* (a), crushed *D. noxia* (b) or wheat leaves after exposure to *D. noxia* (c). Bacterial isolates from this study are shown in red. Only the type *E. iniecta* strain B120 was included in the tree as a representative of the 12 *E. iniecta* strains isolated *(E. iniecta* strains B112, B115, B118, B120, B137, B138, B139, B140, B143, B144, B149, B150). GenBank accession numbers are given in parentheses. Type strains are indicated by a superscript T.

As a second means to determine the contribution of bacteria to aphid virulence and to rule out any possible negative effects of antibiotic use on the aphid feeding, we assessed the

virulence of ‘isolated’ aphids relative to ‘co-fed’ aphids to susceptible wheat plants. Isolation of nymphs from their mothers at birth (‘isolated’) resulted in nearly 100-fold less bacteria than the counts from ‘co-fed’ aphids, where the nymphs were allowed to feed adjacent to their mothers, siblings and progeny ($t_{21} = 8.62$, $P < 0.001$; Fig. 2.3A). After 14 days of feeding, we recovered similar numbers of aphids from susceptible plants exposed to 'isolated' or 'co-fed' aphids ($t_{35} = 1.08$, $P = 0.29$; Fig. 2.3C) showing that the ‘isolated’ aphids were not obviously reduced in fitness during the course of this experiment. Aphids from colonies where individuals were allowed to feed alongside their mothers induced more chlorosis on susceptible wheat plants than did aphids from colonies where individuals were isolated at birth (partial=$F_{1,33} = 20.33$, $P < 0.001$; Fig. 2.3B).

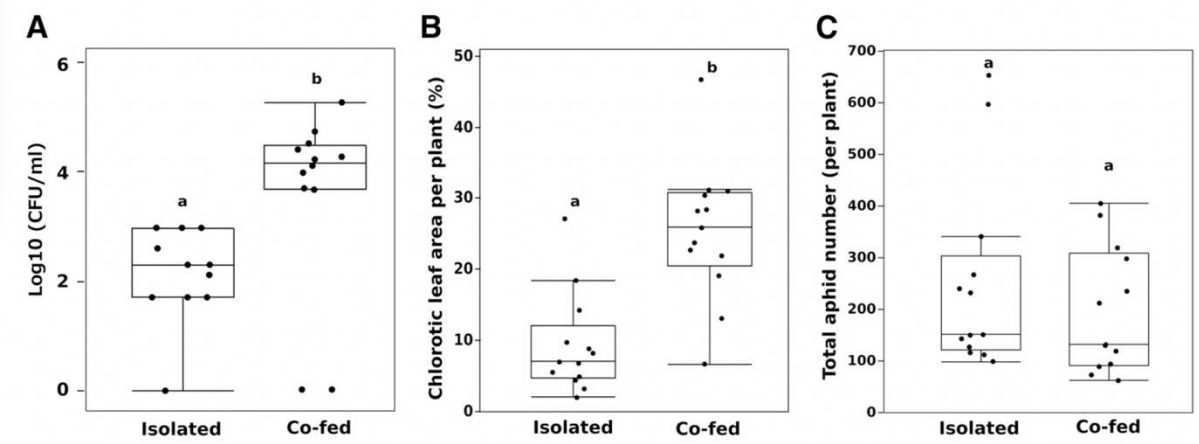

Fig. 2.3. Bacteria associated with *D. noxia* enhance aphid virulence to susceptible wheat. (A) Aphids reared after being ‘isolated’ from their mothers at birth have fewer bacterial-associates then aphids ‘co-fed’, or allowed to feed alongside mothers. (B) ‘Isolated’ aphids caused less chlorosis on susceptible wheat plants at 14 days then ‘co-fed’ aphids. (C) ‘Isolated’ aphids were as fit as ‘co-fed’ aphids at 14 days. For each box plot, circles represent individual measurements from two independent, and are jittered to show all measurements. The central horizontal line corresponds to the sample median. Minimum and maximum observations (not including outliers) are indicated by the horizontal line at either end of the whiskers, while the end of the box represents the interquartile range (between 25% and 75%).

To address if aphid-associated bacteria were the cause of chlorosis, rather than the aphids themselves, wheat leaves were infiltrated with suspensions of the six bacterial groups most frequently isolated from aphids. None of the bacteria tested individually or in combination induced chlorosis or any other phenotype on wheat leaves, showing that the bacteria were not the cause of the chlorotic phenotypes after aphid feeding (Fig. 2.4). Taken together, these support that the presence of bacteria enhances aphid-induced chlorosis on susceptible wheat plants, but that the bacteria themselves are not the cause of chlorosis.

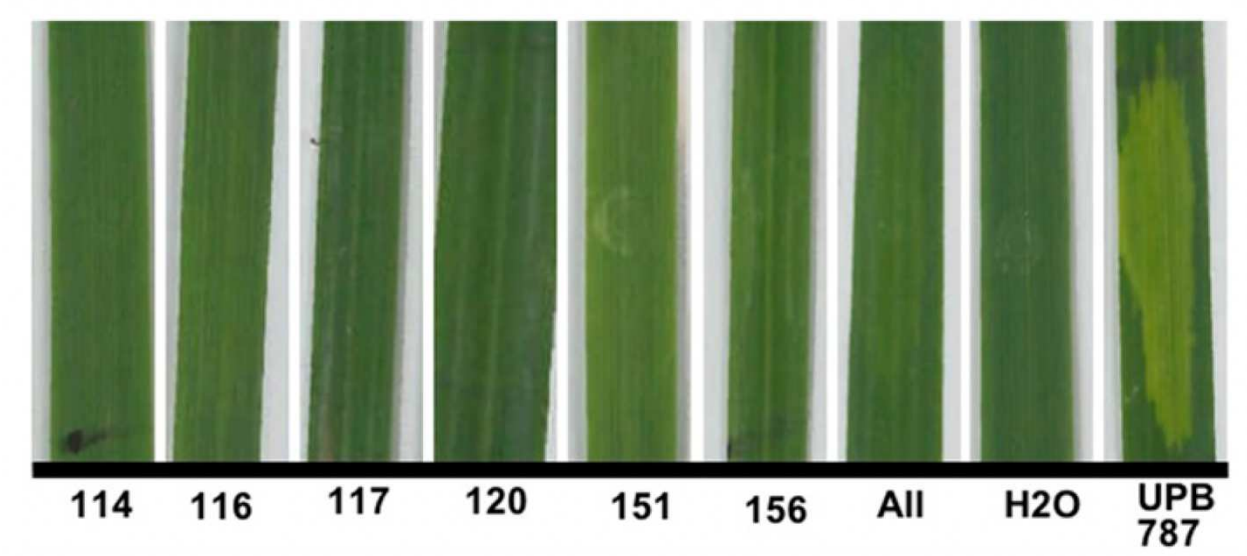

Fig. 2.4. Bacterial strains isolated from *D. noxia* do not induce chlorosis, water soaking, or necrotic symptoms on wheat. Bacterial strains used for inoculations were *Acinetobacter* spp. 114; *Erwinia* sp. 116 and 120; *Arthrobacter* sp.117; *Pantoea* sp. 151; and *Enterobacter* sp. 156. Bacteria were infiltrated into the adaxial surface of plants individually or in combination (All, including equal numbers of each bacterial strain). Controls were water (H2O) and *Xanthomonas translucens* (strain UPB787), a known pathogen of wheat. Only *X. translucens* showed symptoms (water soaking).

'Isolated' aphids harbor fewer *Enterobacteriacae* and *Moraxellacae* than 'co-fed' aphids.

To identify differences in the taxonomic groups of bacterial associates that might contribute to enhanced virulence of 'co-fed' *D. noxia*, we used amplicon sequencing data to compare

microbiomes of 'isolated' and 'co-fed' *D. noxia*. Controls included DNA extractions from samples without aphids (negative control) and a mock community (positive control). Samples without aphids did not amplify with the V4 primers, indicating the samples were free of template DNA (Supplementary Fig. S2.1), and, thus, were not included in downstream analysis. The sequencing of the mock community accurately predicted the 12 bacteria added to the mock community, seven of which were isolated from *D. noxia* in this study (Supplementary Table S2.1), and were thus not included in downstream comparative analyses.

'Isolated' aphid samples harbored bacterial communities with fewer OTUs and lower Faith's phylogenetic diversity than 'co-fed' aphid samples suggesting less diversity, but the differences were not significant (P = 0.86 and 0.18 respectively; Supplementary Fig. S2.6A and B). Bray Curtis's Dissimilarity Matrix analysis showed that bacterial communities from 'isolated' and 'co-fed' aphid samples group separately, indicating differences in bacterial communities (Supplementary Fig. S2.6C).

Members of the families *Enterobacteriacae* and *Moraxellacae* dominated the 'co-fed' aphid microbiome (Figs. 2.5A, 2.6A). Across 'co-fed' samples, up to 80% of the reads remaining after subtraction of *Buchnera* reads belonged to taxa associated with these two families (Figs. 2.5A, 6A). This is in contrast to the 'isolated' microbiome, where taxa associated with *Enterobacteriacea* and *Moraxellacae* were attributed to less than 20% of the non-*Buchnera* reads (Figs 1.5B, 1.6A). Interestingly, one significant sequence variant identified to the family level as *Enterobacteriacae* by ANCOM analysis (Fig. 2.6B) was dominant in 'co-fed' communities but absent from 'isolated' aphid microbial communities (Fig. 2.6A). The sequence was a perfect

match to the 16s rRNA region of *Erwinia iniecta* (Fig. 2.6C). Together, these data show that 'co-fed' aphids are enriched in families of bacterial associates (*Enterobacteriacae* and *Moraxellacae*) that were predicted in the salivary proteome and that were cultured from aphids and aphid-infested wheat (Figs. 2.1 & 2.2). Furthermore, at least one bacterial associate in the *Enterobacteriaceae* is absent in 'isolated' aphid microbiomes, and the sequence matches *E. iniecta,* a bacteria known to be associated with natural populations of *D. noxia* (Campillo et al. 2015; Luna & Leach, unpublished).

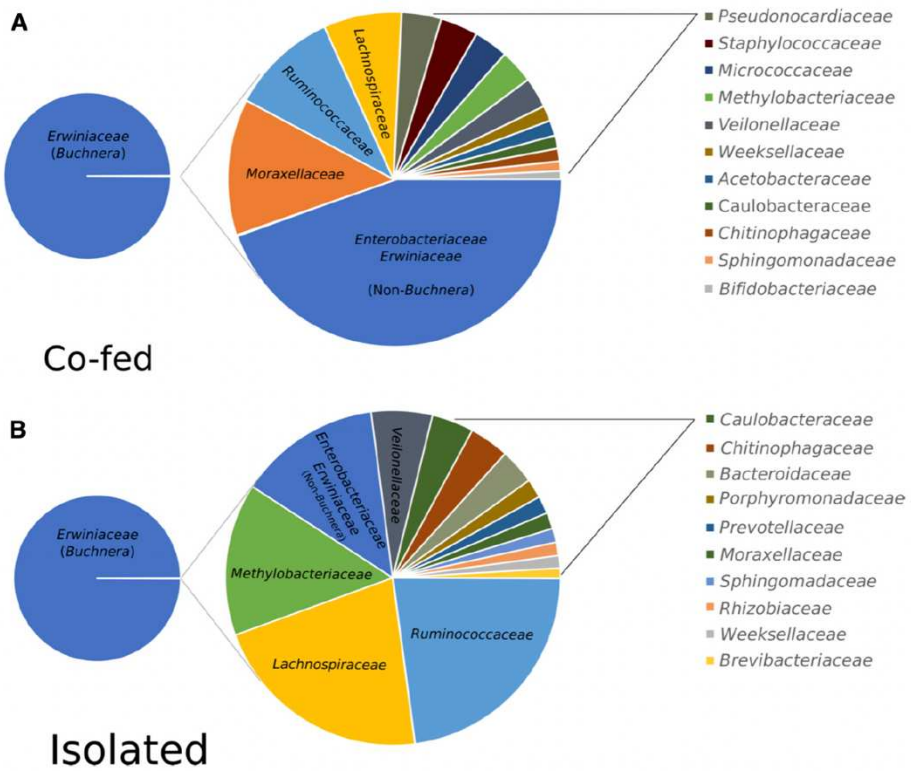

Fig. 2.5 Classification of family-level bacterial taxa associated with *D. noxia*, as predicted by 16s rRNA amplicon sequencing. DNA pools from aphids that were either allowed to feed alongside their mothers, siblings and daughters (Co-Fed) (A), or were isolated from their mothers at birth (Isolated) (B). Over 99% of the 2,052,185 reads obtained mapped to the aphid endosymbiont *B. aphidicola.* Remaining reads from replicated samples for each treatment were averaged.

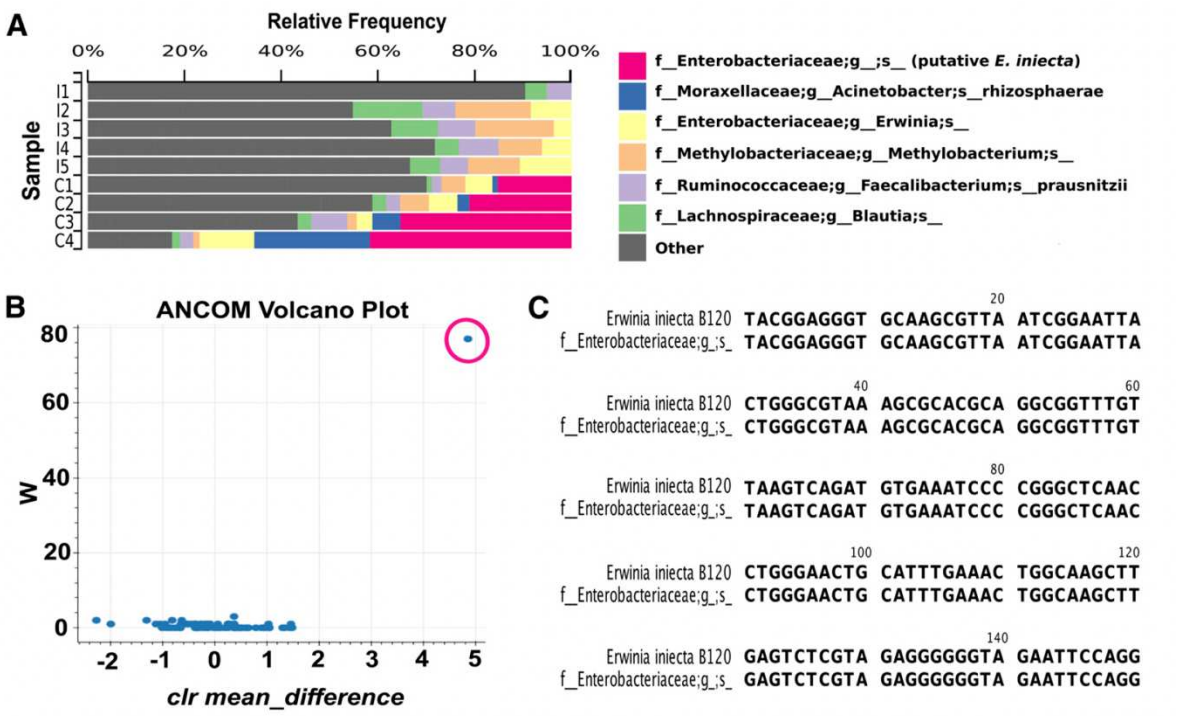

Fig. 2.6. A) Percent abundance of species level taxonomy from 'Isolated' aphids (Samples I1 to I5) and 'Co-Fed' aphids (samples C1 – C4). In legend, f_ represents family and s_ represents species. B) ANCOM (analysis of composition of microbes) volcano plot showing significant sequence variant (circled in pink). C) Nucleotide alignment of significant sequence variant (circled in pink in B) to the 16S rRNA sequence from *Erwinia iniecta*.

***Diuraphis noxia* feeding induces both the JA and SA signaling pathways.** We re-analyzed publicly available data from (Botha et al., 2010) for the effect of aphid colonization ('co-fed' aphids') on wheat genes encoding enzymes from the biosynthetic pathways of the plant hormones salicylic acid (SA) and jasmonic acid (JA), which are known to regulate responses to biotrophic pathogens/sucking insects and chewing insects, respectively. In general, most JA (16/18) and SA (6/6) biosynthetic genes were up-regulated by 'co-fed' aphid infestation at 5 h, in comparison to mock-infested wheat plants (Fig. 2.7A, Supplementary Table S2.5). However, by 48 hpi, most (13/18) JA probe sets were down-regulated relative to mock, while most (4/6)

SA probe sets remain up-regulated. Thus, infection by Russian wheat aphid in wheat leads to an initial induction, followed by a down-regulation of genes involved in JA production, and sustained induction of genes involved in SA production during *D. noxia* feeding.

Accumulation of SA and JA were assessed after infestation of susceptible wheat leaves with 'co-fed' and 'isolated' *D. noxia* to determine if hormone levels were associated with presence of aphid bacterial associates. SA accumulation was induced earlier and to higher levels in plants infested with aphids that harbor higher microbe numbers ('co-fed') relative to aphids with reduced bacterial numbers ('isolated') ($F_{8,18}$ = 54.37, P < 0.0001; Fig. 2.7C). SA did not accumulate over time in plants not attacked by aphids (time*treatment interaction partial-$F_{4,18}$ = 15.69, P < 0.0001). At 24 hpi, SA accumulation in plants treated with 'co-fed' aphids was over four times higher than plants attacked by 'isolated' aphids. At 48 hpi, SA levels in leaves did not differ between infestations with both 'co-fed' and 'isolated' aphids, but was still induced relative to SA levels in uninfested plants (Fig. 2.7C). JA was barely detectable in all samples, and was not significantly different in any treatment relative to controls (Fig. 2.7B). However, trends in JA accumulation are consistent with earlier and higher induction by 'co-fed' aphids relative to 'isolated' aphids, with a reduction to background levels by 48 hpi (ANOVA: $F_{8,18}$ = 0.8670, P = 0.5606). While the trends from JA gene expression and accumulation are not conclusive, our data show that 'co-fed' aphids induce earlier and higher expression and accumulation of SA in wheat leaves than aphids with fewer bacterial associates.

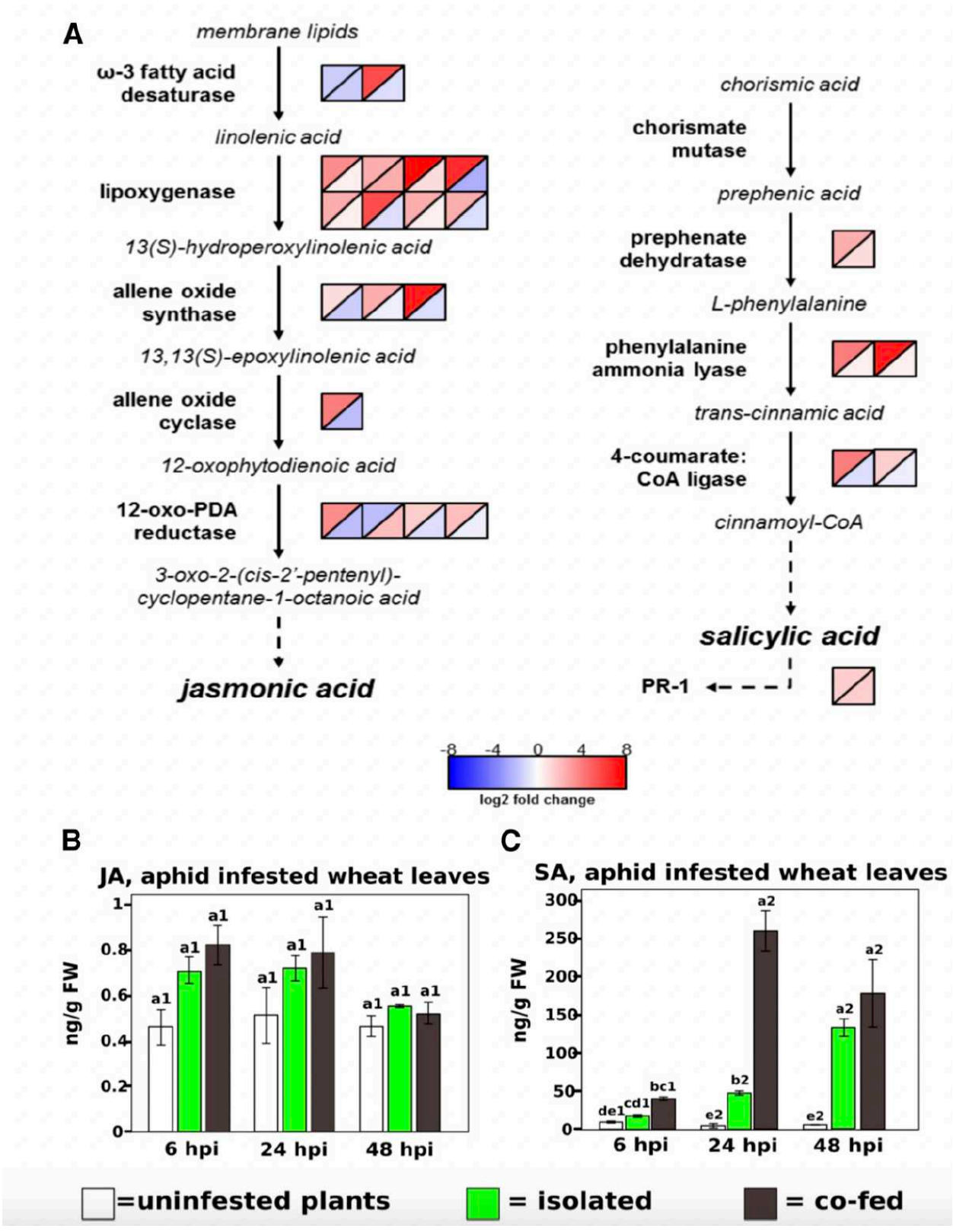
A
membrane lipids
ω-3 fatty acid desaturase
linolenic acid
lipoxygenase
13(S)-hydroperoxylinolenic acid
allene oxide synthase
13,13(S)-epoxylinolenic acid
allene oxide cyclase
12-oxophytodienoic acid
12-oxo-PDA reductase
3-oxo-2-(cis-2'-pentenyl)-cyclopentane-1-octanoic acid
jasmonic acid
chorismic acid
chorismate mutase
prephenic acid
prephenate dehydratase
L-phenylalanine
phenylalanine ammonia lyase
trans-cinnamic acid
4-coumarate: CoA ligase
cinnamoyl-CoA
salicylic acid
PR-1
-8 -4 0 4 8
log2 fold change
B
JA, aphid infested wheat leaves
ng/g FW
6 hpi
24 hpi
48 hpi
C
SA, aphid infested wheat leaves
ng/g FW
6 hpi
24 hpi
48 hpi
=uninfested plants
= isolated
= co-fed

Fig. 2.7. (A) Genes encoding jasmonic acid (JA) and salicylic acid (SA) pathway enzymes in wheat are induced by *D. noxia* biotype 1 feeding. Expression was assessed at 5 and 48 h post-infestation (hpi) of wheat leaves (Botha et al. 2010). Each square represents the $\log_2$ fold change in expression of a probe set ID with homology to a biosynthetic enzyme gene. The top-left half of each square represents expression at 5 hpi, and the bottom-right half represents expression at 48 hpi. Accumulation of (B) JA and (C) SA in susceptible, 14-day-old wheat leaves ('Yuma') 6 and 48 h after infestation treatments of 'isolated' or 'co-fed' aphids or no aphids (uninfested plants). Bars with different letters indicate differences across time and treatment and numbers show differences across time points only. A two-way ANOVA was evaluated the effects of time point (6, 24, or 48 hpi) and aphid treatment (isolated aphids, co-fed aphids, or no aphids). Hormone measurements were log transformed to meet assumptions of normality. Differences between treatment levels were determined using a Tukey's HS.

2. 5 DISCUSSION

In this study, we show that bacteria associated with *D. noxia* facilitate aphid virulence. *D. noxia* release both bacterial proteins and bacteria, mostly *Enterobacteriaceae*, during feeding on artificial diets, as demonstrated by proteome analysis and bacterial cultivation and identification. These aphid-associated bacteria, which were also isolated from aphid-infested wheat and are dominant members of the aphid microbiome, play a role in *D. noxia* virulence, because when bacterial numbers on aphids were experimentally reduced, the aphids caused less chlorosis on wheat.

Previous aphid saliva proteome studies did not report detection of bacterial proteins (Atamian et al., 2013; Bos et al., 2010; Carolan et al., 2011; Cooper et al., 2010; Harmel et al., 2008; Nicholson et al., 2012; Rao et al., 2013; Vandermoten et al., 2014), or, if they did, they revealed only a few matches with obligate endosymbionts such as *B. aphidicola* (Chaudhary et al., 2014; Elzinga et al., 2014). This discrepancy with our findings can be explained by the distinct databases used for the protein comparisons. Studies that did not report any bacterial

proteins used a restricted insect/arthropod database (excluding all other eukaryote/prokaryote proteins) and studies that reported a few endosymbiont proteins used separate insect/arthropod and endosymbiont databases (excluding all other prokaryote/eukaryote proteins). In our study, MS/MS spectra were searched first against the whole non-redundant NCBI database (Fig. 2.1A). This exhaustive comparison showed that most of the identified proteins were of bacterial origin, and many of those were enterobacterial proteins. Some of these proteins identified as 'other Enterobacteria' were predicted to be from various species such as *Escherichia coli*, *Yersinia pestis* or *Salmonella enterica.* Because most identified bacterial proteins are highly conserved across genera (ribosomal proteins, membrane proteins, etc.), and because human pathogens are unlikely to be associated with aphids, their presence was assumed to be an artifact. The lack of specific sequence information for each bacterial species present in the diet necessitated homology-based searching to closely related species. As such, many proteins were identified on the basis of one or a few matching peptides. Therefore, we built a database that included protein sequences from the Aphididae and from species of the four predominant genera identified from aphids, i.e., *Erwinia*, *Pantoea*, *Enterobacter,* and *Acinetobacter*, as well as aphid endosymbionts. Comparisons with this database grouped proteins initially identified as human pathogenic Enterobacteria to one of the four predominant genera (Fig. 2.1B), and provided additional support that the *D. noxia* saliva proteome is mostly comprised of bacterial proteins, especially enterobacterial proteins, with very few proteins from the insect.

Several of the bacterial genera identified as associated with *D. noxia* are typically epiphytic or plant-pathogenic bacteria (Brady et al., 2010; Martinec & Kocur, 1964), although some are

known to associate with insects (Ellers-Kirk & Fleischer, 2006; Estes et al., 2009; Garcia-Salazar et al., 2000; Gitaitis et al., 2003; Hildebrand et al., 2000; Skrodenytė-Arbačiauskienė et al., 2012; Stavrinides et al., 2010; Wells et al., 2002), and, in particular with aphids (Campillo et al., 2015; Capuzzo et al., 2005; Clark et al., 2012; Harada et al., 1997; Plurad et al., 1965). The presence of bacteria from the genus *Erwinia* is not uncommon in aphids. For example, *Erwinia* or *Erwinia*-like bacteria were detected in studies of aphids from the genus *Cinera* as well as in the pea aphid, *A. pisum* (Gauthier et al., 2015; Jousselin et al., 2016). Some bacteria in the taxonomic groups we detected, including *E. iniecta* isolated from *D. noxia*, harbor genes responsible for delivery of bacterial virulence or effector proteins to plant and animal cells (Campillo et al., 2015). Thus, the delivery of these bacteria to plants via aphid feeding, and their requirement for full aphid virulence to wheat plants, begs the question of what the collective role of these microbes is in this multipartite interaction.

None of the bacteria isolated from the aphids or saliva caused damage to wheat leaves when infiltrated individually or in combination (Fig. 2.4). Further experiments with aphids harboring a known and/or controlled microbial flora would be required to identify which individual or combined microbial species facilitate aphids in causing plant damage. Although other facultative endosymbionts that exist in the aphid hemolymph (e.g. *Hamiltonella*, *Regiella*, *Rickettsia*, *Serratia*) are vertically transmitted (e.g. (Chen & Purcell, 1997), it is reasonable to speculate that the culturable bacteria associated with *D. noxia* are transmitted horizontally. Uninfected aphids may acquire bacteria as they co-feed on the same plants where infected aphids had defecated or injected their bacteria-laden salivary secretions. Indeed, such bacteria may be efficiently transferred from mother to offspring in this manner, because by removing

nymphs from their mothers at birth, we were able to establish aphid colonies with reduced bacterial numbers (Fig. 2.3A) and with an altered microbiome composition (Figs. 2.5 and 2.6A). As mother aphids viviparously reproduce, their young aphid offspring may acquire their mother's suite of salivary-bacteria as they feed on the same plant tissue as their nearby mother.

Analysis of the proteome in artificial diets after *D. noxia* feeding has been the subject of studies focused on identifying virulence factors in aphid saliva. Similar to previous proteomic analyses of *D. noxia* saliva, we identified enzymes such as glucose dehydrogenase, trehalase or aminopeptidase N-like and apolipophorin after feeding of both *D. noxia* biotypes 1 and 2 (Cooper et al., 2010; Nicholson et al., 2012). These released proteins have been hypothesized to suppress plant defenses, but this has not been experimentally validated and their role in plant responses remains unclear (Elzinga & Jander, 2013; Rodriguez & Bos, 2013). Despite the fact that aphids transmit ≈ 30% of all plant viruses (Brault et al., 2010), *D. noxia* is considered a poor vector of viruses (Halbert et al., 1994; Summers et al., 1990). Furthermore, no typical aphid effectors have been reported in any proteome studies (Cooper et al., 2013; Nicholson et al., 2012), including ours, and no genes encoding aphid effectors were identified in the *D. noxia* genome (Burger & Botha, 2017; Nicholson et al., 2015). It is interesting to consider whether, in this phytobiome, the lack of aphid salivary effectors, the inability to transmit plant viruses, and the requirement of the bacteria for the aphid to induce damage to plants are linked. In the absence of *D. noxia* effectors, we hypothesize that bacterial effectors or other microbial elicitors such as MAMPs (microbial associated molecular patterns; (Ausubel, 2005; Jones & Dangl, 2006) delivered by the aphid, alter the plant's physiology to favor aphid feeding.

How the bacterial associates contribute to increased aphid virulence is not yet known. Altered regulation of the SA and JA responses have been reported for other plant interactions involving chewing insects (Chung et al., 2013) and stylet-feeding insects (Schwartzberg & Tumlinson, 2014; Su et al., 2015), and bacteria are solely or partially responsible for modulation of the plant hormones in at least some of these interactions. Thus, we tested for variation in SA and JA gene expression and hormone accumulation in *D. noxia* – wheat. With extended aphid feeding (48 h), expression of JA biosynthetic genes that are related to insect defense responses decreased, whereas expression of SA biosynthetic genes that are related to biotrophic pathogen defense responses did not. Further, aphids with experimentally reduced bacterial titers induced less SA accumulation and less damage in wheat plants when compared to aphids with full bacterial titers. Regulation of gene expression and physiological outputs of SA and JA in wheat can be antagonistic (Ding et al., 2016). Although this antagonism is less clear for monocot interactions (Thaler et al., 2012), it is tempting to speculate that the aphid-associated bacteria facilitate aphid feeding by altering hormone regulation.

Insect-microbe associations that influence the impact of plant defenses are likely widespread. Findings from this work will broadly inform us about how such associations function to overcome plant defenses in both wheat as well as other aphid-bacteria-plant systems. Our work highlights that bacteria are key for *D. noxia* to cause damage on susceptible wheat plants, and our preliminary data suggest that the bacteria may help *D. noxia* avoid basal plant defenses. We have not explored if variation in aphid-associated bacteria is related to resistance of wheat to *D. noxia,* or if bacterial content is related to *D. noxia* biotype. Our microbiome analysis and the isolation of aphid-associated microbes are first steps to better

defining which bacteria within this phytobiome are important, and how and when the bacteria and aphids collaborate to counteract or induce different plant defense responses in the context of the wheat plant. Together, these studies may provide novel insights for control of this serious pest of one of the world's staple food crops.

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CHAPTER 3: *ERWINIA INIECTA* SP. NOV., ISOLATED FROM RUSSIAN WHEAT APHIDS (*DIURAPHIS NOXIA*) [2]

3.1 OVERVIEW

Short, Gram-negative-staining rod-shaped bacteria were isolated from crushed bodies of Russian wheat aphid [*Diuraphis noxia* (Kurdjumov)] and artificial diets after Russian wheat aphid feeding. Based on multilocus sequence analysis involving 16S rRNA, *atpD*, *infB*, *gyrB* and *rpoB*, these bacterial isolates constitute a novel clade in the *Erwinia* genus and were most closely related to *Erwinia toletana*. Representative distinct strains within this clade were used for comparisons with related *Erwinia* species. Phenotypic comparisons using four distinct strains and average nucleotide identity (ANI) measurements using two distinct draft genomes revealed that these strains form a novel species within the genus *Erwinia*. Strain B120^T (=CFBP 8182^T= NCCB 100485^T) was designated as the type strain and the name *Erwinia iniecta* sp. nov. is proposed. *Erwinia iniecta* sp. nov. was not pathogenic to plants. However, virulence to the Russian wheat aphid was observed.

3.2 INTRODUCTION

Aphids are sap sucking insects. They penetrate plant tissues using a special mouth part – the stylet– and they probe between the plant cell layers to find sieve elements. Once stylets penetrate the sieve elements, they alternatively inject saliva and suck the plant sap (for review see (Miles, 1999). This process for obtaining nutrients establishes an intimate and long term

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interaction between the aphids and the host plants. In a few cases, aphids are proposed to vector or deliver plant pathogenic bacteria into the plants via their stylets (Plurad et al., 1965; Stavrinides et al., 2009; Watanabe et al., 1996).

In a previous study, we repeatedly and consistently isolated *Enterobacteriaceae* from sterile artificial diets (15% aqueous sucrose, pH 7.2) fed on by Russian wheat aphids [*Diuraphis noxia* (Kurdjumov)] (unpublished results, T. Campillo, L. van Eck, E. Luna, N. Lapitan, N. Tisserat, J.E. Leach). The bacteria were isolated only from artificial diets exposed to probing of *D. noxia* sylets through a parafilm membrane; no bacteria were isolated from sterile diets that had not been exposed to aphids. In addition, bacteria were isolated from crushed aphid bodies and from wheat exposed to *D. noxia* feeding. Based on 16S rRNA sequences of 30 diverse isolates from these various origins, most of the bacteria grouped within the *Enterobacteriaceae.*

Of the bacteria isolated from aphid bodies or artificial diets or wheat after feeding by aphids, we found a subset of isolates that belong to the *Erwinia-Pantoea* clade. While *Erwinia* and *Pantoea* species are most commonly epiphytic or plant pathogenic bacteria (Brady, Venter, et al., 2010; Martinec & Kocur, 1964), various members of this genera also are known to associate with insects (Ellers-Kirk & Fleischer, 2006; Estes et al., 2009; Garcia-Salazar et al., 2000; Gitaitis et al., 2003; Hildebrand et al., 2000; Skrodenytė-Arbačiauskienė et al., 2012; Stavrinides et al., 2010; Wells et al., 2002), and especially aphids (*Hemiptera*: *Aphididae*) (Bansal et al., 2014; Capuzzo et al., 2005; Clark et al., 2012; Harada et al., 1997; Plurad et al., 1965). The aim of the present work was to characterize the *Erwinia-Pantoea* isolates associated with *D. noxia* and released during feeding.

3.3 MATERIAL AND METHODS

The *D. noxia*-associated bacteria characterized in this study were isolated by inoculation on Eosin Methylene Blue agar (EMBa) medium (Holt-Harris & Teague, 1916) and incubation for 48 h at 28°C. One strain (B137) was isolated from *D. noxia* (biotype 1) whole bodies, previously decontaminated 5 min in 70 % ethanol, and crushed into 100 µl Carlson's solution (Harada et al., 1996). The solution was used to inoculate EMBa plates. Three additional strains (named B120^T, B149 and B150) were obtained from sterile artificial diets (15 % sucrose aqueous solution, pH 7.2) presented to approximately 500 adult aphids under a parafilm layer as previously described (Cooper et al., 2010). After 48 h feeding, the diet solution was centrifuged, and the resuspended pellet was used to inoculate EMBa. No bacteria were isolated from the artificial diets that were not exposed to aphid probing, showing that the bacteria were released into the diets by aphid feeding. All bacterial strains were purified three times on EMBa medium to obtain pure cultures. The origin of each strain is detailed in Table 3.1.

Table 3.1. Strains used in the present study and their origins.

Strains	Isolation	Reference
Erwinia iniecta		
B112	Surface decontaminated wheat leaf fed by *D. noxia* biotype 2	This study
B115	Surface decontaminated wheat leaf fed by *D. noxia* biotype 1	This study
B118	Surface decontaminated crushed *D. noxia* biotype 2	This study
B120^T (= CFBP 8182^T = NCCB 100485^T)	Artificial diet fed by *D. noxia* biotype 2	This study
B137 (= CFBP 8183 = NCCB 100486)	Surface decontaminated crushed *D. noxia* biotype 1	This study

B138	Surface decontaminated crushed *D. noxia* biotype 1	This study
B139	Surface decontaminated crushed *D. noxia* biotype 1	This study
B140	Surface decontaminated crushed *D. noxia* biotype 1	This study
B144	Surface decontaminated crushed *D. noxia* biotype 1	This study
B149 (= CFBP 8184 = NCCB 100487)	Artificial diet fed by *D. noxia* biotype 2	This study
B150 (= CFBP 8185 = NCCB 100488)	Artificial diet fed by *D. noxia* biotype 2	This study
Erwinia amylovora CFBP 1232^T	Wilt disease on *Rosaceae*	(Hauben et al., 1998)
Erwinia aphidicola CFBP 6829^T	Pea aphid (*Acyrthosiphon pisum*) gut	(Harada et al., 1997)
Erwinia oleae CFBP 8201^T	Olive knots	(Moretti et al., 2011)
Erwinia toletana CFBP 6631^T	Olive knots	(Rojas et al., 2004)
Erwinia typographi CFBP 8202^T	Bark beetle (*Ips typographus*) gut	(Skrodenytė-Arbačiauskienė et al., 2012)
Escherichia coli K-12 MG1655	Human feces	(Demerec & Fano, 1945)
Pseudomonas syringae* pv. *syringae B106	Turkish Filbert	(Ibarra et al., 2012)

^{T}= type strain

Whole genome sequencing was performed on DNA from two selected isolates (B120^T and B149). Genomic DNA was extracted using an Easy DNA kit (Invitrogen) according to the

manufacturer's instructions, and DNA sequencing was performed on an Illumina MiSeq (2 x 250 bp run format on a full MiSeq v2 flowcell) by the Research Technology Support Facility of Michigan State University (East Lansing, MI). After trimming and high quality sequence read selection using Trimmomatic (v0.27), a total of 1,697,366 reads with an average 214 bp read length were obtained for B120^T, and 2,721,395 reads with an average 218 bp read length were obtained for B149. The average coverage for B120^T and B149 was equivalent to 75.9 x and 124.4 x, respectively. De novo assembly was performed with Velvet (v1.2.08). Manually optimized parameters allowed us to obtain 101 contigs (maximum length = 259,650 bp; N50 = 139,128 bp) totaling 4,790,664 bp for B120^T and 142 contigs (maximum length = 292,162 bp; N50 = 123,532 bp) totaling 4,781,836 bp for B149.

Sequences of the housekeeping genes *atpD*, *gyrB*, *infB* and *rpoB* and the 16S rRNA of strains B137 and B150 were obtained from polymerase chain (PCR) fragments. PCR was performed using 5x PCR Phusion HF buffer (Finnzymes), 0.2 mM dNTP, 0.5 mM forward and reverse primers (listed in Table S3.1), 25 ng extracted genomic DNA, 3 % DMSO, and 0.5 unit of Phusion Taq polymerase (Finnzymes) in 20 µl aqueous solution. An initial denaturation step was performed at 98°C for 1 min followed by 35 cycles: 10 s at 98°C, 30 s at the annealing primer temperature and 1 min at 72°C. The reaction ended with a final extension step of 4 min at 72°C. Amplified housekeeping genes were sequenced in both directions by the Colorado State University Proteomics and Metabolomics Facility (Fort Collins, CO) after a purification step using a Wizard SV Gel and PCR Clean-Up System (Promega).

3.4 RESULTS AND DISCUSSION

The B120^T and B149 16S rRNA sequences were obtained from the genome sequence (by BLAST to the *E. amylovora* CFBP 1232^T 16S rRNA sequence), and were 1,497 nucleotides each. The B137 and B150 16S rRNA sequences were obtained after PCR amplification (Primers listed in Table S3.1); after sequencing, 1,360 and 1,382 nucleotides, respectively, were obtained. Sequences of overlapping regions obtained from B120^T, B149, B137, and B150 were identical. Comparison of these sequences to the Genbank/EMBL/DDBJ database using BLAST searches showed that they shared the highest percentage identity to 16S rRNA from the type strains *Erwinia toletana* LMG 24162^T (99 %), *E. persicina* LMG 11254^T (98 %), *E. billingiae* LMG 2613^T (98 %), *E. aphidicola* LMG 24877^T (98 %) and *Pantoea calida* 1400/07^T (98 %).

Using CLUSTALW and the software MEGA version 5 (Tamura et al., 2011; Thompson et al., 1994), the 16S rRNA sequences of isolates B120^T, B137, B149 and B150 were aligned with the available sequences of sixteen *Erwinia* species type strains, five *Pantoea* species type strains, three *Tatumella* type strains and *Escherichia coli* (strains and accession numbers are detailed in Table S3.2).

This alignment allowed building two phylogenetic trees based on maximum likelihood (Fig. 3.1) and neighbor joining (*data not shown*) methods (Saitou & Nei, 1987; Tamura & Nei, 1993). In both trees, the four new isolates clustered together into the clade *Erwinia-Pantoea*. This affiliation was confirmed by the fifteen *Erwinia*-specific nucleotide positions in the 16S rRNA sequences, as described by Hauben et al. (1998). Positions of these “nucleotide signatures” are indicated in the species description.

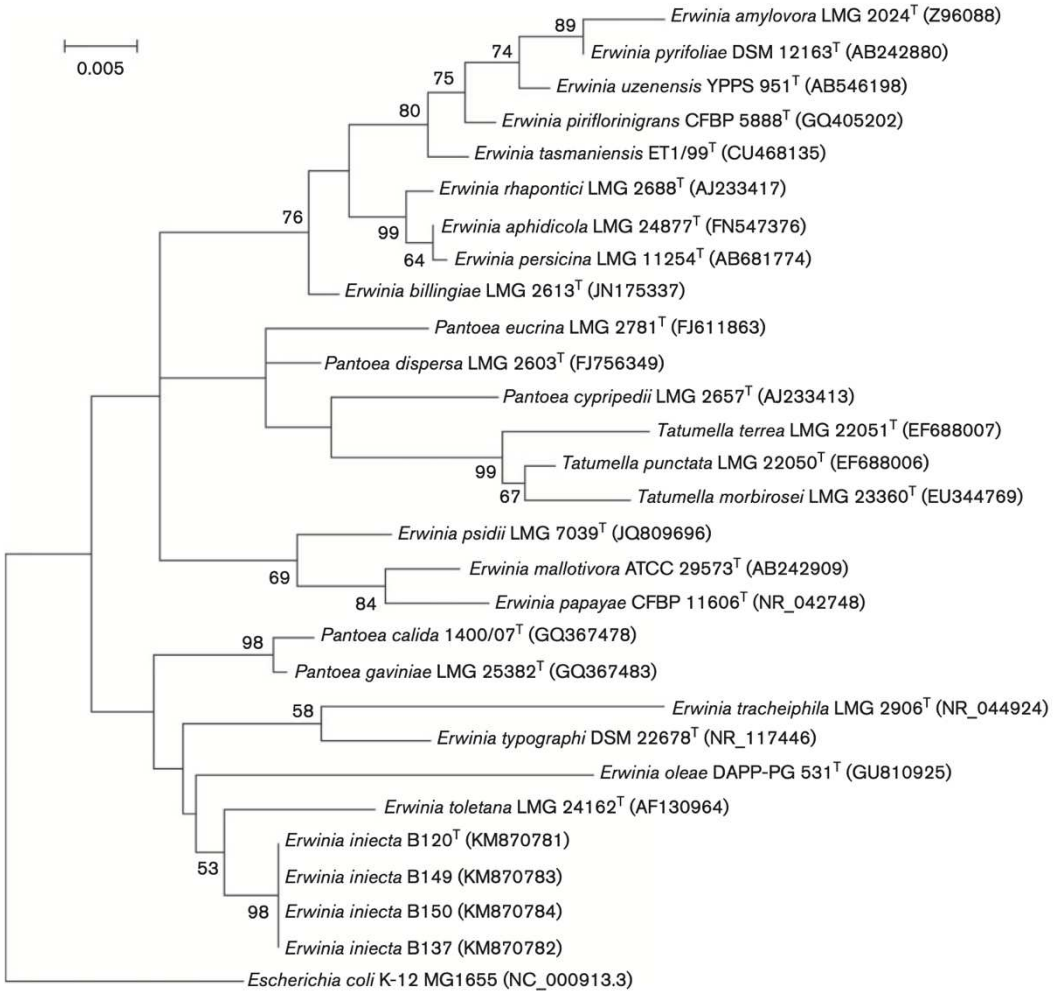

Fig. 3.1 Maximum likelihood phylogenetic tree based on partial 16S rRNA gene sequences. Bootstrap values indicated at nodes are based on 1000 replications. The bar represents 0.5 % nucleotide substitutions. GenBank accession numbers are given in parentheses. *Escherichia coli* is the outgroup.

To clearly delimit the clade constituted by isolates B120^T, B137, B149 and B150, a multilocus sequence analysis (MLSA) was performed with a concatenate of the 16S rRNA plus four housekeeping genes (*atpD*, *gyrB*, *infB* and *rpoB*) that were previously used for deriving the *Erwinia* taxonomy (Brady et al., 2012; Brady, Cleenwerck, et al., 2010; Popp et al., 2010). Using either the maximum likelihood method (Fig. 3.2) or the neighbor joining method (*data not shown*), the MLSA clearly separated the three *Erwinia, Pantoea* and *Tatumella* genera, as

previously shown (Brady, Cleenwerck, et al., 2010; Brady, Venter, et al., 2010; Popp et al., 2010). The new monophyletic group composed by B120^T, B137, B149 and B150 was supported by high bootstrap values, and it unambiguously belongs to the *Erwinia* genus. The cluster was clearly distinct from its closest relatives, *E. toletana*, suggesting it was a novel species. The MLSA also showed diversity among the new isolates in the analyzed housekeeping genes. B120^T and B149 were distinct from B137 and B150 by at least five polymorphic sites and B137 was distinct from B150 by one polymorphic site. This confirms our analysis was based on at least three distinct strains.

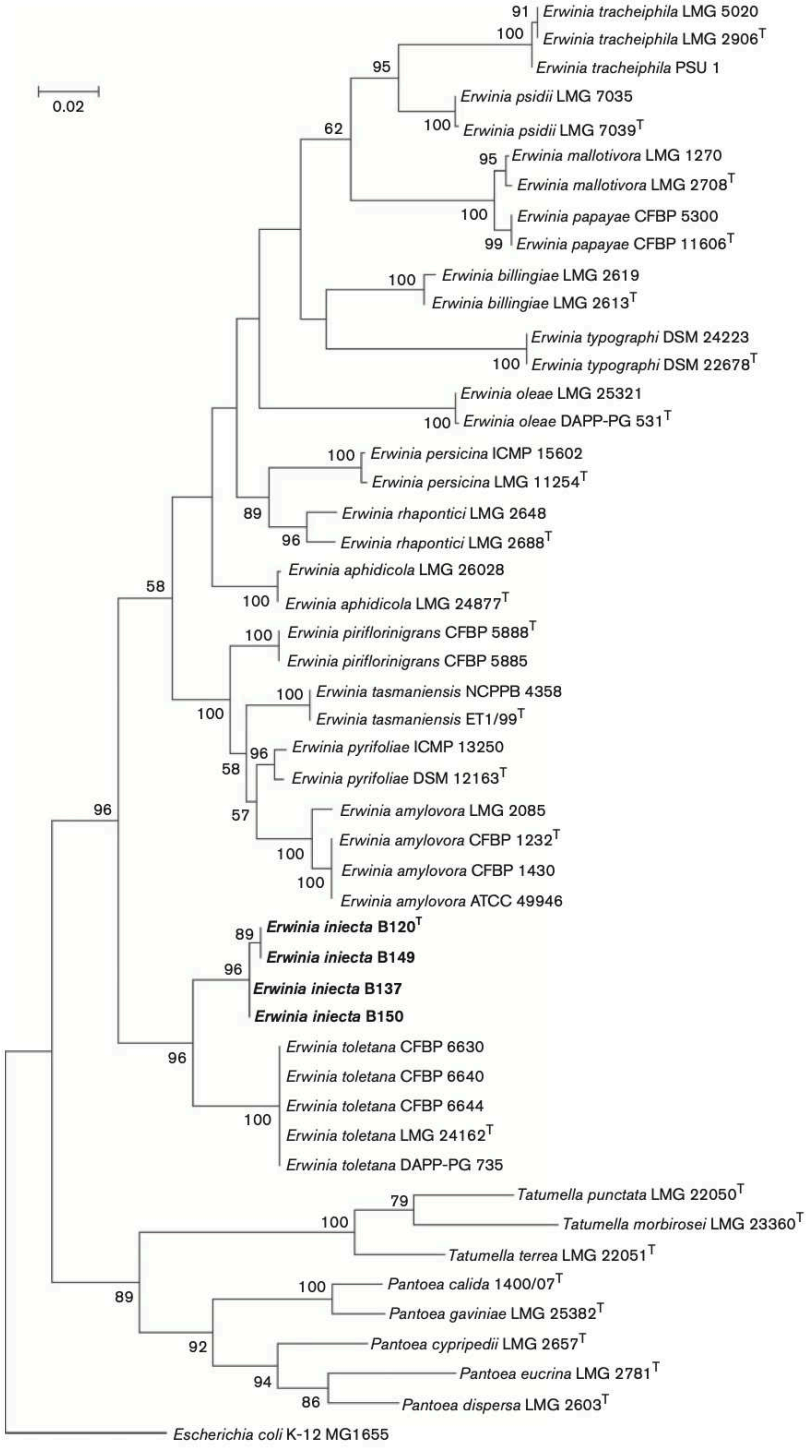

Fig. 3.2 Maximum likelihood phylogenetic tree based on partial 16S rRNA, atpD, gyrB, infB and rpoB gene sequences. Bootstrap values indicated at nodes are based on 1000 replications. The bar represents 2 % nucleotide substitutions. *Escherichia coli* is the outgroup.

The G+C contents of B120^T and B149 were 52.21 % and 51.08 %, respectively. The genome comparison with related strains allowed measurement of the average nucleotide identity (ANI), a tool for bacterial species delineation that replaces DNA-DNA hybridization (Goris et al., 2007; Richter & Rosselló-Móra, 2009). The B120^T sequence (used as query) was compared with nine *Erwinia* genome sequences, including seven different known species using the software Jspecies set on default parameters for Blast-based analysis (ANIb). Our previous MLSA study showed *E. amylovora* ATCC 49946, *E. amylovora* CFBP 1430, *E. toletana* DAPP-PG 735 and *E. tracheiphila* PSU 1 were close to their species type strain (less than 0.002 base substitutions per site, Fig. 3.2). Therefore, these strains were used for the ANI evaluation, even though they were not type strains (type strain sequences of *E. toletana, E. tracheiphila* or other *Erwinia* species were not available). The ANI percentages between B120^T and the *Erwinia* strains were between 76.16 % and 77.38 % (Table 3.2). The accepted ANI cut-off that distinguishes two bacterial species (corresponding to the 70 % DDH) is 95 % (Richter & Rosselló-Móra, 2009). To explore this cut-off within the *Erwinia* genus, ANI values were calculated among *E. amylovora* CFBP 1232^T, *E. billingiae* CFBP 6830^T, *E. piriflorinigrans* CFBP 5888^T, *E. pyrifoliae* CFBP 4172^T and *E. tasmaniensis* Et1/99^T (Table S3.3). The ANI values calculated among these type strains ranged between 75.59 % and 90.20 %, and at least six couples of type strains had an ANI values higher than the value obtained between B120^T and its closest neighbor *E. toletana* DAPP-PG 735 (77.38 %). Taken together these results suggested B120^T does not belong to the species *E. toletana* or to any other tested species.

Table 3.2. Percentages of Average Nucleotide Identity (ANI) between *E. iniecta* sp. nov. strain B120^T and other *Erwinia* available genome sequences.

Species	Strain	Accession number	% ANI with B120^T
Erwinia amylovora	CFBP 1232^T	GCA_000367625.2	76.08
Erwinia amylovora	ATCC 49946	FN666575-7	76.16
Erwinia amylovora	CFBP 1430	FN434113	76.16
Erwinia billingiae	LMG 2613^T	NC_014306	77.02
Erwinia piriflorinigrans	CFBP 5888^T	CAHS01000001–25	76.18
Erwinia pyrifoliae	CFBP 4172^T	NC_017390.1	76.42
Erwinia tasmaniensis	Et1/99^T	NC_010694.1	76.17
Erwinia toletana	DAPP-PG 735	NZ_AOCZ00000000	77.38
Erwinia tracheiphila	PSU 1	NZ_APJK00000000	74.95
Erwinia iniecta sp. nov.	B149	JRXF00000000	99.8
Erwinia iniecta sp. nov.	B120^T	JRXE00000000	100

^{T} Type strains

The draft genomes of B120^T and B149 were uploaded on the SEED server and genes were delimited and annotated using the Rapid Annotation using Subsystem Technology (RAST) service (Aziz et al., 2008). About 4,324 and 4,329 coding sequences were predicted for B120^T and B149, respectively, allowing prediction of functions commonly found in *Erwinia* genomes. Both strains harbored three different type III secretion systems that were similar to the well-described *Erwinia* type III secretion systems encoded by the pathogenicity islands PAI-1 (*hrp* genes), PAI-2 and PAI-3 (*inv*/*spa* genes) (Smits et al., 2010). Genes encoding type III secretion systems clustered with known chaperone and translocator protein-encoding genes like *dspEF*, suggesting these secretion systems are involved in pathogenicity (Bogdanove et al., 1998).

Automatic annotation suggested the presence of genes necessary for flagellar synthesis (*flgABCDEFGHIJKLMN*; *fliACDEFGHIJKLMNOPQRSTZ* and *flhCD*), antibiotic resistance (genes *bcr*, *uppP*, *pmrHFIJKLM* and *sanA* conferring, respectively, resistance to bicyclomycin, bacitracin, 68olymyxin and vancomycin), arabitol and acetoin/butandiol degradation (*budRABC*) and Quorum sensing (*sdiA, phzI* and *luxS*).

Pathogenicity of B120^T, B137, B149 and B150 to wheat TAM 107 (*Triticum aestivum* L.) and tobacco (*Nicotiana benthamiana*) was tested using three different inoculation methods (leaf infiltration, leaf clipping and needle injection), and was assayed in three independent experiments. After 4 h incubation in Luria Broth (LB) medium at 28°C (exponential growth phase), strains were suspended in 0.8 % NaCl at an optical density 600 nm of 0.1 (5 X 10^7 CFU.ml^{-1}). Bacterial suspensions were directly infiltrated into 7-day-old wheat leaves or 30- day-old tobacco leaves using a 1 ml needle-less syringe. Inoculations by leaf clipping with scissors dipped in bacterial suspensions and by injection of the bacterial suspension into the first node using a syringe with a needle were also performed on wheat. After inoculation, plants were incubated at 24°C with a 16-8 h day-night photoperiod and 85 % humidity for 21 days. No aberrant phenotypes suggestive of disease were caused by any of the four isolates in inoculated seven-day-old wheat leaves after 15 days incubation (*data not shown*). In addition, 30-day-old tobacco leaves inoculated by infiltration did not show any symptoms (disease or a hypersensitive response, as induced by *Pseudomonas syringae*) after 21 days. These data suggest that strains of the new *Erwinia* species are not pathogenic to plants.

Strains B120^T and B137 were tested for virulence to *D. noxia*. Viability of *D. noxia* feeding on artificial diets supplemented with individual *E. coli* K-12, B120^T or B137 at a final

concentration of 1 X 10^8 CFU.ml^{-1} or no bacteria, was monitored daily by counting living and dead aphids over 11 days. Artificial diets with aphids feeding on them were incubated at room temperature under fluorescent light for 12 h per day. Three independent replications (each one containing three technical repetitions) were performed. *D. noxia* populations that were fed on *E. coli* K-12, B120^T or B137 decreased from 50 to five aphids in six days or less (Supplementary Fig. S3.1). In contrast, all of 50 *D. noxia* fed on a diet without any bacteria remained alive over the 11 days. During the first two days, 90, 50 and 30 % of the *D. noxia* populations died after feeding B137, B120^T and *E. coli* K-12, respectively. These experiments confirm *E. coli* K-12 is an aphid pathogen when ingested (Altincicek et al., 2011) and show B137 and B120^T are more virulent than *E. coli* K-12.

Using API strips 20E, 20NE and 50CH (BioMérieux), 64 different phenotypic traits of the novel strains B120^T, B137, B149 and B150 and the strains *E. toletana* CFBP 6631^T, *E. oleae* CFBP 8201^T, *E. amylovora* CFBP 1232^T, *E. aphidicola* LMG 24877^T and *E. typographi* CFBP 8202^T were analyzed. Tests were read visually after 48 and 72 h incubation at 25°C. Complementary tests were performed using Biolog GN microplate system (GEN III plate) at the Colorado State University veterinary diagnostic laboratory (http://csu-cvmbs.colostate.edu/vdl). Carbon source utilization was observed in each well by five measurements at OD_{590} taken between 15 and 48 h after bacterial strains had been incubated in the plate at 28°C (Table S3.4). The data we obtained for the type strains of *E. toletana, E. oleae*, *E. amylovora*, *E. aphidicola* and *E. typographi* matched with their original descriptions (Table 3.3, (Brady, Cleenwerck, et al., 2010; Harada et al., 1997; Moretti et al., 2011; Rojas et al., 2004; Skrodenytė-Arbačiauskienė et al., 2012). Three of the four novel strains (including the type strain B120^T) exhibited the exact same

profile for all tests. One strain, B149, differed for only two tests; this strain was the only strain to degrade potassium gluconate and potassium 5-cetogluconate. These novel strains were differentiated from their closest relative *E. toletana* by their ability to degrade D-arabitol, D-cellobiose, raffinose and gentiobiose, their lack of ability to degrade citrate, their ability to produce acetoine, and their ability to reduce nitrate (Table 3.3). Phenotypic characteristics of the new *Erwinia* species are provided in the species description below.

Table 3.3. Selected phenotypic characteristics differentiating *E. iniecta* sp. nov. from other *Erwinia* species**.** +, positive; – , negative; nd, not determined.

Characteristic	***E. iniecta* sp. Nov.** (B120^T, B137, B149, B150)	***E. toletana*** CFBP 6631^T	***E. oleae*** CFBP 8201^T	***E. amylovora*** CFBP 1232^T	***E. aphidicola*** CFBP 6829^T	***E. typographi*** CFBP 8202^T
Utilization of:						
Amygdalin	+	+	–	nd	+	–
Arbutin	+	+	–	–	+	–
Citrate	–	+	–	nd	+	–
D-adonitol	–	–	–	–	+	–
D-arabitol	+	+	+	–	–	–
D-arabinose	–	–	+	–	–	–
D-cellobiose	+	+	–	–	+	–
D-fucose	+	+	–	–	+	nd
D-lactose	+	+	–	–	+	–
D-lyxose	–	–	–	–	+	–
D-maltose	+	+	–	–	+	–
D-mannose	+	+	+	–	Nd	+
D-melibiose	+	+	–	–	+	+
D-raffinose	+	–	–	–	+	–
D-sucrose	+	+	–	+	–	–
D-xylose	+	+	–	–	+	+
Erythritol	–	–	–	–	+	–
Esculin	+	+	+	–	+	–
Gentiobiose	+	–	–	+	Nd	–
Glycerol	+	+	– [c]	–	+	–
Potassium gluconate	–	–	+	–	+	–
Potassium 2-cetogluconate	–	–	+	–	+	–

Raffinose	+	– [a]	–	–	+	–
Salicin	+	+	–	–	+	–
Xylitol	–	–	–	–	+	–
Production of:						
Acetoine	+	–	–	+	+	–
Nitrate reduction	+	– [a]	+	–	+ [b]	+

[a] Data from Maria Rojas et al., 2004

[b] Data from Harada et al., 1997

[c] Data from Moretti et al., 2011

Fatty acid (FAME) analysis was performed by the MicrobialID company (Newark, DE). Strain B120^T was grown in Luria agar at 28°C for 48 h and its fatty acid profile was determined using the MIDI Sherlock Microbial Identification System (www.microbialID.com). The four most abundant fatty acids were C16:0 (32.0 %), 18:1ω7c (18.8 %), 16:1 ω7c and/or 16:1 ω6c (17.5 %) and 17:0 cyclo (10.3 %), accounting for around 78 % of the total fatty acids.

In conclusion, based on 16S rRNA sequence comparison, multilocus sequence analysis and phenotypic tests, the four isolates B120^T, B137, B149 and B150 are distinct from each other, but that they belong to the *Erwinia* genus, in the *Enterobacteriaceae* family. Combined with the average nucleotide identity analysis, we propose that these strains represent a novel *Erwinia* species.

Species description of Erwinia iniecta sp. nov.

Erwinia iniecta (in.iec'ta. L. fem. Part. Adj. *iniecta* means thrust-in, injected). The strains isolated from the artificial media were introduced or 'thrust in' via the aphid stylet.)
Cells are rod shaped (0.5-0.7 x 1.5-2 µm), gram negative, aerobic, motile and non-spore-forming. After 24 h at 28°C on nutrient agar, cells form light-beige, circular, small (between 1 and 2 mm), with entire margins, and translucent colonies. No specific pigment (diffusible or fluorescent) was observed in. The optimal growth temperature is 28°C. Growth occurs at 37°C but not at 42°C. Strains grow in nutrient broth in the presence of 1 %, but not 4 %, NaCl. They are negative for gelatin liquefaction and hydrogen sulfide production but positive for acetoin production. They exhibit catalase, tryptophan deaminase and nitrate reductase but do not exhibit β-galactosidase, oxidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, nitrite reductase and tryptophanase.

E. iniecta strains degraded the following carbon sources: D-sucrose, D-melibiose, amygdalin, D-xylose, D-mannose, arbutin, esculin, salicin, D-cellobiose, D-maltose, gentiobiose, D-raffinose, D-lactose, D-arabitol, glycerol, D-fucose. However, up to 48 h they were unable to degrade: citrate, D-adonitol, D-arabinose, D-lyxose, erythritol, and xylitol. B120^T, B137, B149 and B150 exhibited the same profile for all tests except for potassium gluconate and potassium 5-cetogluconate degradation tests; only strain B149 was able to degrade these two compounds. *E. iniecta s*trains are not pathogenic on wheat or tobacco. Strains are pathogenic to Russian wheat aphid (*D. noxia*) when ingested.

E. iniecta harbors specific *Erwinia* signature nucleotides in 16S rRNA gene sequence (Hauben et al., 1998): A408, A594, C598, G639, G646, C839, G847, G987, G988, C989, G1216, C1217, C1218, C1308 and G1329, according to the *Escherichia coli* 16S rRNA gene sequence numbering (Brosius et al., 1981).

G+C content is 52.21 % and 51.08 % for B120^T and B149, respectively. The predominant fatty acids are: C16:0 (32.02 %), 18:1ω7c (18.75 %), 16:1 ω7c and/or 16:1 ω6c (17.52 %), 17:0 cyclo (10.30 %), 14:0 3OH and/or 16:1 iso I (9.42 %), 14:0 (4.79 %) and 12:0 (4.03 %).

The type strain B120^T (=CFBP 8182^T = NCCB 100485^T) and the strains B137 (=CFBP 8183 = NCCB 100486), B149 (=CFBP 8184 = NCCB 100487) and B150 (=CFBP 8185 = NCCB 100488) were isolated in Colorado from surface decontaminated *D. noxia* bodies and artificial diets fed by *D. noxia*, biotype 1 or 2. Additional strains of this species were also isolated from ground, surface-decontaminated wheat leaves.

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CHAPTER 4: BACTERIA ASSOCIATED WITH RUSSIAN WHEAT APHID HONEYDEW MODULATE DEFENSE RESPONSE GENE PATHWAYS IN BARLEY

4.1 INTRODUCTION:

Aphids have a long evolutionary history of symbiosis with bacteria, dating back approximately 160 to 280 million years ago (Fukatsu, 1994; Moran et al., 1993). One of the most well characterized primary symbionts is the obligate bacterium, *Buchnera aphidicola. B. aphidicola* resides in specialized cells called bacteriocytes, which collectively form the bacteriome, and are located near the aphid gut. *B. aphidicola* plays a crucial role in aphid nutrition by synthesizing essential amino acids that are lacking in the aphid's phloem-based diet (Baumann, 2005; Douglas & Prosser, 1992). In addition to primary symbionts, aphids also harbor facultative (secondary) symbionts, which contribute to a range of adaptive benefits, including enhanced fecundity (Castañeda et al., 2010; Chen et al., 2000), resistance to heat stress (Russell & Moran, 2006), and protection against pathogens or parasitoids (Oliver et al., 2003; Scarborough et al., 2005). While facultative symbionts are not essential for aphid survival, they play crucial ecological roles, particularly under environmental stress or biotic interactions.

The Russian wheat aphid (*Diuraphis noxia,* RWA) is a major pest of wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*) and other small grains. It is distributed across all major wheat-growing regions, and has recently established in Australia (Jankielsohn, 2013; Yazdani et al., 2018). Climate models predict continued expansion of RWA into new regions as global temperature rise (Jing et al., 2023). Severe infestations can result in up to an 80% yield loss on wheat and barley, with economic impacts in the United States alone exceeding one billion dollars since the insect's introduction (Jing et al., 2023).

Control strategies for RWA include chemical pesticides, biological control and host plant resistance. In wheat and barley, resistance is conferred by single resistance genes. Unfortunately, aphid populations evolve and overcome defenses regulated by single *R* genes as a result of the evolution of new biotypes (Nicholson et al., 2012). In the United States, biotype 2 is the most virulent, overcoming the *Dn4* resistance gene (Haley et al., 2004). The mechanisms underlying this adaptation remain unknown, but effector-mediated suppression of plant immunity is suspected (Elzinga & Jander, 2013; Nalam et al., 2019; Smith & Boyko, 2007). However, RWA lacks well-characterized salivary virulence effectors, raising questions about how it successfully suppresses plant defenses (Botha, 2021; Botha et al., 2005; Luna et al., 2018; Nicholson et al., 2012).

Plant defense responses to biotic stresses, such as pathogen infection or herbivory by chewing and piercing-sucking insects, are primarily associated with changes in levels of the plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA). SA is typically associated with defenses against biotrophic pathogens, while JA is more commonly linked to necrotrophic pathogens and chewing insects. Aphids have evolved diverse strategies to manipulate these hormonal pathways, with outcomes varying depending on the aphid species and the host plant's susceptibility.

Many stylet feeding insects, including RWA, induce JA-related defenses in resistant plant varieties (Nalam et al., 2012; Smith et al., 2009). In some cases, stylet feeders can suppress JA signaling to promote feeding. This suppression of JA is thought to occur through induction of SA, which antagonizes JA-mediated defenses (Zarate et al., 2007).

Among SA-regulated defense components, thionins are small cysteine-rich antimicrobial peptides that are rapidly induced in response to aphid feeding and have been associated with plant defense against both pathogens and insects (Sharma et al. 2021). In barley, thionins have been identified as early responders during aphid attack and are functionally linked to the pathogenesis-related (PR) gene family, reinforcing their role in SA-mediated resistance (Sharma et al. 2021, Escudero-Martinez et al 2017).

Other studies suggested that aphid-associated microbes might influence plant susceptibility, but the underlying mechanisms remain unclear (Luna et al., 2018). For example, our previous research detected bacterial proteins in the saliva of RWA, raising the possibility that these bacteria contribute to aphid virulence (Luna et al., 2018). RWA populations with high numbers of bacteria caused significantly greater damage to plants and induced SA at six times greater than aphids with reduced bacterial numbers (Luna et al., 2018). The relevance of JA was less clear, as although JA genes were induced early then declined, we were unable to detect JA accumulation. Thus, our previous work predicted the involvement of SA in RWA-plant interactions and suggested a role for microbes in its induction, however, the specific source and mechanism of microbe-driven effects remain unclear.

Given previous observations that RWA-associated microbes are linked to plant defense response modulation, we sought to determine the source of microbes that induce plant defense responses. The aphid stylet would be a likely tool to introducing the microbes to the plant's intercellular spaces, and the detection of microbes in artificial diets probed by RWA during feeding suggested this as a source of defense-activating microbes. However, others have found that infiltration of leaves with aphid honeydew induces plant defense responses

(Schwartzberg, 2014) and given that leaves are heavily contaminated with honeydew during an aphid infestation, it is possible that the stylet becomes contaminated during feeding and can carry the microbes to the internal plant parts. We report here that based on Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and culturing studies, the RWA honeydew is the likely source of plant defense-inducing microbes, and that based on RNA-sequencing (RNA-Seq) studies, the honeydew-associated microbes control barley defense responses partially through regulation of expression of phytohormone biosynthesis and regulatory genes.

This research bridges knowledge gaps on plant-aphid-microbe interactions, defines how microbial transmission through honeydew affects host plant defenses, provides molecular evidence linking aphid-associated bacteria to altered plant defenses allowing increased aphid aggressiveness on susceptible plants.

4.2 MATERIALS AND METHODS

Biological Material: Plant growth, aphid rearing and bacterial strains

Colonies of *D*. *noxia* were reared and maintained as described (Luna et al., 2018; Randolph et al., 2009). Briefly, RWA biotype 2, originally collected in Colorado from wheat field infestations, was maintained on wheat plants (*Triticum aestivum* L.) cultivar Byrd and barley plants (*Hordeum vulgare*) cultivar Morex. Plants were grown in a greenhouse in 4-inch plastic pots under controlled conditions: a temperature of 23 ± 3 °C, a relative humidity of 25%, and a photoperiod of 16 h light and 8 h dark. Aphids were contained in thrips-proof insect cages (BugDorm-4E3074) to prevent unintended infestations or cross-contamination with other

aphids. Two populations of aphids were maintained: isolated aphids contained fewer bacteria, and co-fed aphids harbored a full suite of bacteria (Luna et al., 2018). Bacterial cultures of *Winslowiella iniecta* (strain B120) were plated on nutrient agar and incubated at 28 °C for 24 h.

Microscopy

Scanning Electron Microscopy

Aphid samples were fixed in 2.5% glutaraldehyde 0.15 M in Sorensen's phosphate buffer (pH 7.0) at 22 °C for 30–60 min, followed by storage at 4 °C. Samples were dehydrated through a graded ethanol series and then subjected to final dehydration using a Bio-Rad E3000 critical point dryer (Quorum Technologies). They were then sputter coated with 10 nm gold layer and imaged at 5 kV using a JEOL JSM6500F Field Emission Scanning Electron Microscope. Images were saved as tiff files.

Transmission Electron Microscopy

Samples were fixed similarly to the method described above, except that 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) was used, with a post fixation step in 1% osmium tetroxide in 0.1M sodium cacodylate buffer.

Honeydew collection, microbial quantification and infiltration

For experiments utilizing honeydew, wheat plants were infested with aphids and after 2 weeks (Supplementary Fig. S4.1A), the plants were shaken over a series of sieves (No. 16, 35 and 45, respectively; Supplementary Fig. S4.1B) to filter out large aphids and debris. The pan was then gently moved in a circular motion to separate honeydew from smaller aphids

(Supplementary Fig. S4.1C). While a small number of debris remained in the final honeydew product, samples were free of aphids (Supplementary Fig. S4.1D).

For microbial quantification of honeydew, 1 mL of sterile distilled water was added to 50 mg of honeydew in a 1.5 mL collection tube. Samples were incubated for 1 h and then sonicated for 2 min to dislodge microbes that may have adhered to the honeydew particles and to help solubilize samples. Serial dilutions (1:10) were prepared, and 5 µL of each dilution was plated on nutrient agar. Plates were incubated at 28°C for 48 h, after which colony-forming units (CFUs) were counted.

To compare CFUs/ml between treatments, we performed a Mann-Whitney U test (Wilcoxon rank-sum test), using RSTUDIO (V2024.04.2+764). This non-parametric test was chosen due to the non-normal distribution of CFU/ml values.

To prepare the honeydew infiltrate, 150 mg of honeydew was suspended in 5 ml of sterile distilled water and incubated and sonicated as described above. The honeydew solution was then filtered through a 4 µm (Caporaso et al., 2011) syringe filter, which allowed the passage of bacteria while removing larger particles. In experiments involving a honeydew infiltrate, sterile distilled water was used as a negative control.

Honeydew Microbiome Sequencing and Analysis:

For the honeydew microbiome analysis, honeydew was collected as previously described from co-fed and isolated aphids. Negative isolation controls were included to assess contamination. DNA was extracted using the DNeasy PowerSoil Kit (Qiagen) following the

manufacture's protocol with the modification that samples were diluted in 20 µL of elution buffer. The V4 region of the 16S rRNA gene was amplified using 515F and 806R primers (Caporaso et al., 2011). Amplicons were purified, quantified and pooled in equimolar concentrations. Library preparation, pooling, quality control and sequencing was performed at the Colorado State University Next Generation Sequencing Facility. Sequencing was conducted on an Illumina MiSeq v2 system with 2x250 bp paired-end reads, generating 20,000-30,000 reads per sample. Due to sequencing limitations, three biological replicates were included for each treatment, except for the co-fed aphid samples, where only two biological replicates were sequenced due to sequence failure of one sample. Sequenced data was processed using MicrobiomeAnalysist using default parameters (Dhariwal et al., 2017). MicrobiomeAnalyst employs methods such as PERMANOVA that are robust to unequal group sizes.

Experimental Design for Transcriptomics

Two parallel experiments were conducted for transcriptomic analysis: one with honeydew-infiltrated plants and the other with RWA-infested plants. In the honeydew infiltration experiment, plants were infiltrated with one of three treatments: (1) honeydew solution collected from isolated aphids, which contained lower titers of bacteria; (2) honeydew solution collected from co-fed aphids, which contained higher bacterial titers; or (3) a negative control, in which plants were infiltrated with water. In the RWA infestation experiment, plants were either (1) infested with isolated RWAs, (2) infested with co-fed RWAs, or (3) left untreated as a negative control. For both experiments, each of the three treatments were assessed at four timepoints (6, 24, 48 and 72 h) with three biological replicates per timepoint. This resulted in 36

plants per experiment. Samples from each treatment and timepoint combination were collected for downstream analyses.

At the time of seeding, a plastic vented sleeve was added to the soil to prevent unintended herbivore attacks (Supplementary Fig. S4.2 C). Plants were treated at the two-leaf stage (Supplementary Fig. S4.2 B). For the honeydew infiltration experiment, approximately 50 µL of a honeydew solution was infiltrated into the adaxial side of the youngest fully expanded leaf. For the RWA-infestation experiment, approximately 0.5 g of aphids (≈2,500 aphids) were placed inside a 1×1-inch piece of vinyl tubing positioned around the plant (Supplementary Fig. S4.2 A). The vinyl tubing was attached to a bamboo skewer and positioned at the leaf sheath base, and a small foam piece was placed at the bottom to secure the aphids in the tube.

Before the start of the experiments, bacterial concentrations were determined in the honeydew and in the RWA colonies. For tissue collection in the RWA experiment, the youngest leaf was excised using scissors, and aphids were gently brushed off using a soft-bristled paintbrush. Leaf tissue was placed in a 2 mL collection tube, immediately frozen in liquid nitrogen, and later pulverized using a TissueLyser (Qiagen). Samples were stored at -80C until RNA extraction.

For the quantitative real-time PCR experiment, 14-day-old barley plants were infiltrated with, honeydew or *Winslowiella iniecta*. Honeydew infiltration was performed as described above. For the *W. iniecta treatment,* approximately 50 µL of a *W. iniecta* suspension (1×10^8 CFU ml^{-1}) was infiltrated into each leaf (Luna et al., 2018; Reimers & Leach, 1991), and the

infiltrated area was outlined with a permanent marker. Leaf tissue from the infiltrated area was collected at 6, 24 or 48 h for RNA extraction.

RNA extraction, cDNA synthesis and RNA sequencing

Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen ID: 74904) following the manufacturer's instructions and including a DNase treatment. RNA quantity and purification were assessed using a NanoDrop 2000 spectrophotometer and TapeStation 4150 (Agilent Technologies) to determine the RNA Integrity Number (RIN). For the transcriptomic experiment, 1 ug of high-quality RNA (RIN ≥ 6.2) was sent to Novogene (Novogene, Sacramento, CA, USA) for RNA sequencing on the Illumina NovaSeq 6000 platform. Libraries were prepared from messenger RNA (mRNA) and sequenced to obtain 150-bp-paired-end reads.

Transcriptomic analysis, differential expression

Raw Illumina sequence reads in FASTQ format were quality assessed using FastP (v0.23.4) and MultiQC (v1.27). High-quality reads were then used for transcript quantification performed with Salmon (v0.6.0), using the quasi-mapping-based mode (Patro et al., 2017). A transcriptome index was constructed from the reference genome (Morex V3 downloaded from e!DAL – Plant Genomics and Phenomics Research Data Repository at https:edal-pgp.ipk-gatersleben.de/)(Mascher, 2021). The output provided transcript-level abundance estimates in transcript per million (TPM) and read counts which were summarized to gene-level counts using tximport package implemented in DESeq2 (Love et al., 2014). Genes with low counts were filtered out (10 counts in at least 50% of samples). Variance-stabilizing transformation (VST)

was applied to filtered dataset to generate expression matrix for downstream analysis. iDEP 2.0 was used for exploratory analysis of the data (Ge et al., 2018) and the DESeq2 package (Love et al., 2014) was used to perform differential expression analysis with a threshold of false discovery rate (FDR) <0.1 and a minimum fold change of two. Gene ontology (GO) overrepresentation analysis was conducted using PANTHER (v19) (Mi et al., 2017). The "GO biological process complete" annotation set for *Hordeum vulgare* sp. *vulgare* was used. Fisher's exact test was performed, and FDR was calculated to correct for multiple testing.

Weighted Gene Co-expression Network Analysis

To identify the gene co-expression patterns associated with plant responses to treatment of honeydew with high (co-fed) or low (isolated) bacterial numbers a weighted gene co-expression network analysis (WGCNA) was performed in R Studio (v2024.04.2+764) using the WGCNA package (v.1.73) Gene expression similarity was assessed using Pearson's correlation coefficients, and a signed network was constructed. A soft-threshold power of 14 was used to achieve a scale-free topology. The adjacency matrix was converted into a topological overlap matrix (TOM), and hierarchical clustering of TOM-based dissimilarity identified modules via the dynamic tree cut method with a minimum module size of 30 genes. Modules with eigengene correlations above 0.7 were merged. Relationships between module eigengenes and bacterial treatments were evaluated with Pearson's correlation and associated p-values (≤0.05). Hub genes were identified as those with high intramodular membership (top 15% of module membership scores) or the highest weights in the modules of interest. Module-

specific gene lists were extracted, and network files were exported to visualize intramodular networks in VisANT (Hu et al., 2008).

Honeydew Enrichment Experiment

One hundred honeydew pellets collected from either isolated or co-fed colonies were added to 5 ml of sterile nutrient broth and incubated at 28 °C for 24 h. After incubation, samples were centrifuged, and the supernatant was discarded. DNA was extracted from the resulting pellet using the Blood and Tissue DNA extraction kit (Qiagen). DNA was amplified using primers specific to the RWA-associated microbe, *W. iniecta*.

Primer development for *Winslowiella iniecta*

UniqPrimer (Karim et al. 2019) was used to design primers for the bacterium *W. iniecta*. The "include" genomes, used to identify unique target regions, were *W. iniecta* (B120 and B149), while the "exclude" genomes, which ensured selected regions were absent in non-target genomes, included strains frequently isolated from RWA: *Erwinia* sp. B116, *Pantoea* sp. B151 and *Enterobacter* sp. 156 (Luna et al. unpublished).

Quantitative Real-Time PCR Validation

Quantitative real-time PCR (qRT-PCR) was performed to validate trends in expression of hormone-responsive genes in response to bacterial treatments. Total RNA was extracted from barley leaf tissues using RNeasy Plant Mini Kit (Qiagen ID: 74904) and residual genomic DNA was removed following the manufactures' instruction. First-strand cDNA was synthesized from 1 µg of total RNA using Superscript reverse transcriptase (ThermoFisher Scientific) according to

the manufacture's protocol. The qRT-PCR reactions were carried out using PerfeCTa SYBR Green SuperMix (QuantaBio) on a BioRad CFX96 with three technical replicates per biological replicate. Primers were designed to amplify *Actin* (FW: ACCTTCAGTTGCCCAGCAAT; RV: CAGAGTCGAGCACAATACCAGTTG; gift from Jonathan Jacobs, Ohio State University) and *Thionin* [FW: TATGGCCAAGGTCGTTTTGT; RV: CATAACTAAGATGATACATTTGCTTCG; *HvTHIO1* (Escudero-Martinez et al., 2017)]. Barley actin was used as the reference gene to normalize gene expression levels.

Raw Cq values from technical replicates were averaged to obtain a single value per biological replicate. The ΔCt was calculated by subtracting the reference gene Cq from the target gene Cq. ΔΔCt values were calculated by comparing each treatment group to a calibrator sample, and relative gene expression was determined using the 2^ΔΔCt method. Mean fold-change in expression was calculated from biological replicates for each treatment and time point.

4.3 RESULTS:

Bacterial Localization on Russian Wheat Aphids (RWAs)

To determine where microbes associated with RWA are located, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) studies were conducted. SEM revealed the presence of bacteria on the stylet sheath and mandibular stylets of adult aphids raised under co-fed conditions (Fig. 4.1A-B). In TEM images, there was no evidence of bacterial colonization in the internal structures of the mouth parts, such as the primary and auxiliary

salivary glands (Fig. 4.1C). These findings suggest that bacteria delivered into plants do not originate from inside the aphid stylet or mouthparts.

As aphids feed, they deposit copious amounts of honeydew on the plant leaf surface (Fig. 4.1D). During feeding on honeydew-fouled leaves, aphid stylets likely become contaminated with microbes present in honeydew, providing a means to deliver microbes into the plant tissues. Therefore, we addressed the possibility that honeydew could be a source of bacteria that interact with plant cells during RWA infestation.

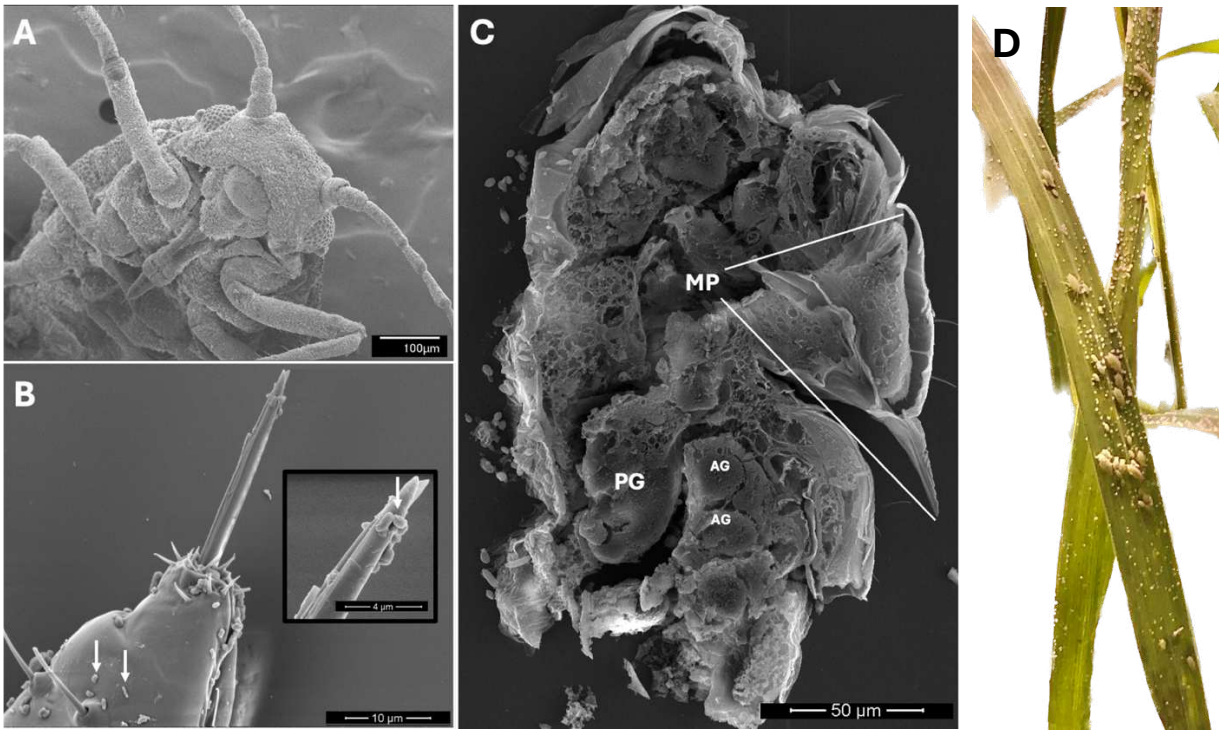

Fig. 4.1 A-D: Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of adult Russian wheat aphid (RWA). **(A)** Supine view of RWA. **(B)** White arrows show the presence of bacteria on the stylet sheath (labium) and mandibular stylets (inset). **(C)** Longitudinal central section of aphid head showing the mouthparts (MP), primary salivary gland (PG), and auxiliary salivary glands (SG). **(D)** RWA feeding on leaf surrounded by honeydew.

Bacterial Composition of RWA Honeydew

Honeydew collected from RWAs (Supplementary Fig. S4.1) contained bacteria, with significantly higher bacterial titers in co-fed aphid honeydew compared to isolated aphid honeydew (Supplementary Fig. S4.3A; $P < 0.0001$). Taxonomic analysis at the family level revealed a predominance of Enterobacteriaceae in honeydew from both aphid groups, with contributions from other families such as Staphylococcaceae, Bacillaceae, Streptomycetaceae and other unclassified bacteria (Fig. 4.2B). Species richness (Chao1 index) was not significantly different between honeydew from isolated and co-fed aphids ($p = 0.657$; t-test statistic = -0.504, Supplementary Fig. S4.3A), suggesting that differences in the microbiome of the two RWA populations is related to shifts in community composition rather than the number of species. A PCoA plot based on Bray-Curtis dissimilarity showed a clear separation of microbial communities between honeydew collected from isolated and co-fed aphids (PERMANOVA $F = 9.21$; $R^2 = 0.754$; $p = 0.1$, Supplementary Fig. S4.3B). Species-level taxonomy showed diverse representation of genera such as *Cronobacter*, *Pantoea*, *Staphylococcus* and uncultured bacteria. Only a small percentage of the RWA endosymbiotic bacteria *Buchnera aphidicola* was detected in the co-fed treatment (Supplementary Fig. S4.3C).

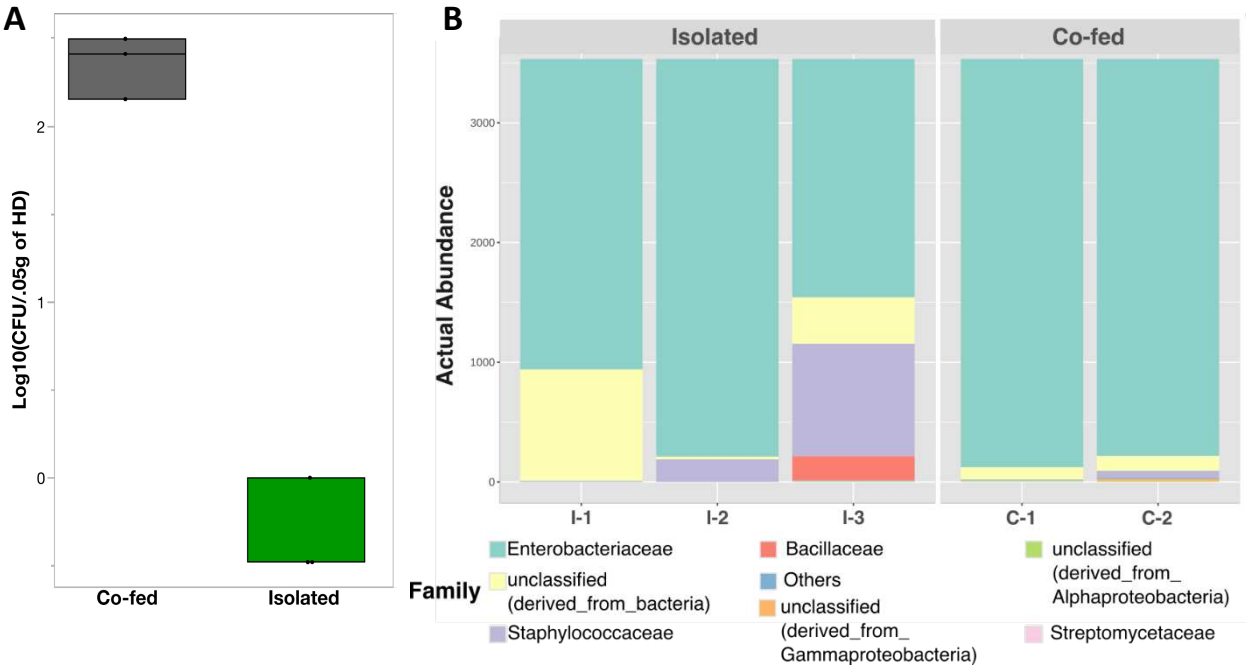

Fig. 4.2: RWA honeydew contains bacteria. **(A)** Bacterial titers (Log10 CFU/5 mg honeydew) are higher in honeydew isolated from co-fed aphids compared to honeydew from isolated aphids. **(B)** Family level taxonomy of RWA honeydew reveals a high relative abundance of bacteria from the family Enterobacteriaceae in honeydew collected from both co-fed and isolated aphids. Samples I1-3 and C1-2 represent replicates of 'Isolated' and 'Co-fed' treatments, respectively.

Transcriptomic Responses to Honeydew Treatments

To determine transcriptional responses in barley to bacteria associated with RWA honeydew, we conducted RNA sequencing of leaves infiltrated with honeydew collected from isolated or co-fed aphids and examined transcript profiles at 6, 24, 48 and 72 h post honeydew infiltration. A total of 2.4 billion reads were obtained, with 22 million removed after filtering. On average, Phred scores were 36 and the GC content was 59% indicating high-quality RNA sequencing data. Sequenced reads were mapped to the barley (*Hordeum vulgare*) reference

genome (Morex V3) with an average mapping rate of 71%. This approach identified transcripts corresponding to 18,742 genes.

Differentially expressed genes (DEGs) in co-fed compared to isolated treatments were identified at each timepoint. At 6 h, 159 genes were upregulated and 52 genes were downregulated. At 24 h 20 genes were upregulated and 23 genes were downregulated. The highest number of DEGs occurred at 48 h with 1777 genes upregulated and 364 genes downregulated. At 72 h, 940 genes were upregulated, and 497 genes were downregulated (false discovery rate [FDR] <0.1, fold ≥ 2, p ≤ or equal to 0.05). Thus, the infiltration of honeydew collected from co-fed aphids, the microbe-enriched honeydew, triggers a stronger transcriptomic response in barley compared to infiltration of honeydew collected from isolated aphids, particularly at later timepoints.

A set of 641 DEGs was upregulated at 48 and 72 h followed by 27 upregulated DEGs co-expressed at 6 and 48 h. Most upregulated DEGs were unique to the later timepoints, with 1105 upregulated DEGs unique to 48 h and 284 upregulated DEGs unique to 72 h (Fig. 4.3B). Although alternative splicing events are common in plant transcriptomes, this study focused on gene-level differential expression. Analysis of transcript-level splicing events was not conducted, as it was beyond the scope of this project but could be explored in future studies.

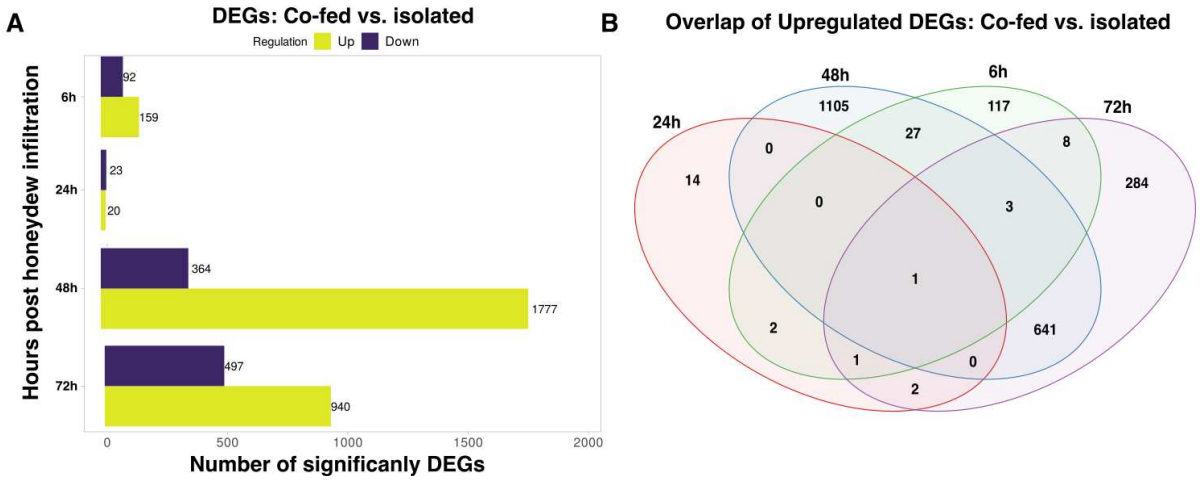

Fig. 4.3. (A) Differentially expressed genes (DEGs) in plants infiltrated with honeydew from co-fed RWA compared to honeydew from isolated aphids at 6, 24, 48, and 72 h. Yellow indicates genes that are differentially upregulated, and purple indicates genes that are differentially downregulated. **(B)** Overlap of upregulated DEGs across time points in response to honeydew from co-fed aphids compared to isolated aphids. The Venn diagram shows shared and unique DEGs at 6, 24, 48 and 72 h.

Module-Trait Relationships Identified by WGCNA

To reduce data dimensionality, identify patterns of gene expression, and group genes into modules that reflect biological processes, a Weighted Gene Co-expression Network Analysis (WGCNA) was performed using all expressed genes from the transcriptomic analysis. By linking gene co-expression modules to phenotypic traits, WGCNA revealed specific biological networks involved in barley's transcription. These modules showed co-expression patterns associated with honeydew that was enriched with bacteria (from the co-fed RWA).

WGCNA identified 15 modules, with colors assigned randomly. The Salmon module was significantly correlated with the co-fed honeydew treatment (p=0.05), indicating that genes in this module are highly co-expressed and expressed in response to the honeydew enriched in

microbes. The Black module showed a trend toward significance at p= 0.08, showing moderate co-expression in response to this treatment (Fig. 4.4).

The Salmon module contained 1549 genes, of which 836 were DEGs. Notably, 54% (833) of these DEGs were upregulated and only a few were downregulated (Table 1). This pattern illustrates that this module serves as a key driver of transcriptional changes in response to co-fed honeydew. The Black module was comprised of 1930 genes, of which 33% (645) were DEGs, with nearly all (643) being upregulated. Although not significant at $p \leq 0.05$, the Black module may contribute to the plant's transcriptional responses to co-fed honeydew.

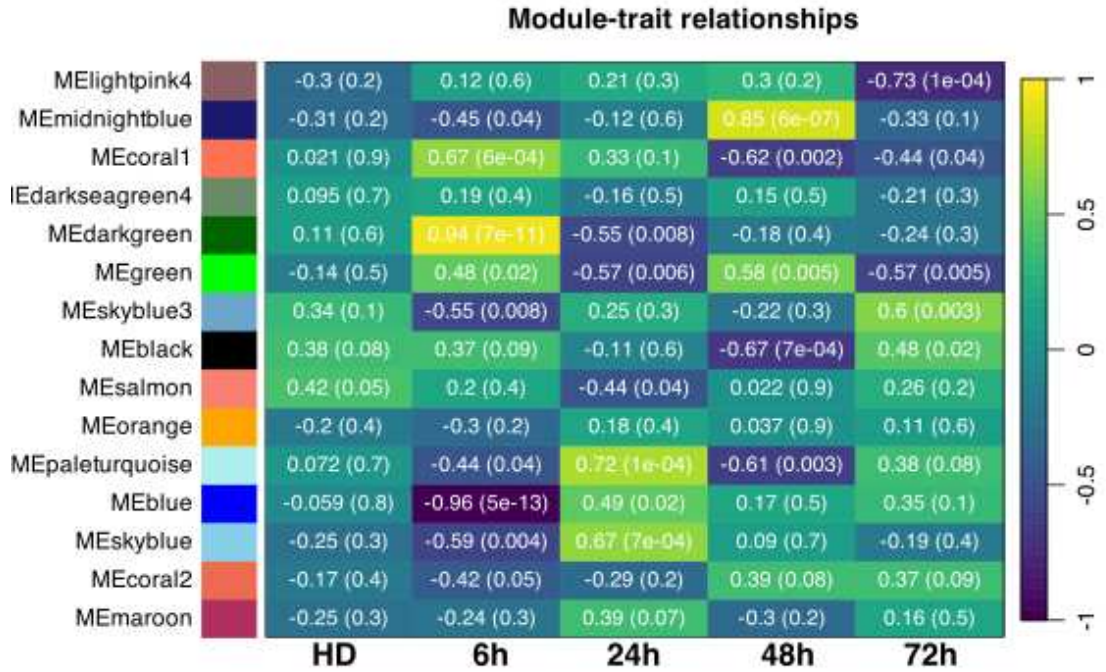

Fig. 4.4. Module-trait relationships identified by Weighted Gene Co-expression Network Analysis (WGCNA). Pearson correlation coefficients (first value) and corresponding p-values (in parentheses) for each module eigengene (rows) and trait (columns). Modules with significant positive correlations are in yellow and stronger negative correlations are in purple. The "HD" column corresponds to the co-fed compared to isolated honeydew treatment across all timepoints.

Table 1: Overview of gene expression in WGCNA modules.

Module	Total Genes	Total DEG	Upregulated DEG	Downregulated DEG
Lightpink4	214	36	1	36
Midnightblue	3068	216	7	209
Coral1	611	106	35	74
Darkseagreen4	59	8	8	0
Darkgreen	3645	507	108	403
Green	1420	104	20	85
Skyblue3	140	49	49	0
Black	1930	645	643	2
Salmon*	1549	836	833	22
Orange	235	3	0	3
Paleturquoise	2277	233	116	122
Blue	2525	187	76	119
Skyblue	1028	124	6	119
Coral2	47	2	0	2
Maroon	87	0	0	0

*Module is significant at p = 0.05

Functional Enrichment and Co-Expression Network Analysis of Key Modules

To further explore the functional relevance of the Salmon and Black modules, we analyzed the enriched Gene Ontology (GO) biological processes for upregulated DEGs in each module. For the Salmon module, we identified hub genes and constructed a co-expression network of key hub genes.

DEGs in Salmon and Black modules are overrepresented in plant defense-related GO terms (Fig. 4.5 A,B). In the Salmon module, processes such as “defense response to bacterium”, “defense response to oomycetes”, “chitin catabolic process”, “defense response to fungus”, and several receptor-mediated defense signaling and recognition processes were significantly overrepresented. These findings suggest that genes in the Salmon module play a central role in microbial recognition and activation of defense response pathways in barley (Fig. 4.5A). In the Black module, “apoptotic process” and “chorismate biosynthetic process” were among the plant defense-related processes identified (Fig. 4.5B).

We identified 232 hub genes in the Salmon module based on high module membership and gene significance (Table S1). Among these were many genes related to plant defense responses, including those encoding 44 protein kinases, six WRKY transcription factors, two NB LRRs, two ethylene-responsive transcription factors, a jasmonate-ZIM-domain protein, an abscisic acid-responsive protein, and phenylalanine ammonia-lyase. Gene expression in the Salmon module peaks around 48 h, with a more pronounced response in the co-fed honeydew treatment compared to the isolated treatment (Supplementary Fig. S4.4). Among all hub genes identified, we further pinpointed 10 that demonstrated the strongest connections among all co-expressed genes within the Salmon module, many of which were also associated with plant defense responses (Table 2) including a receptor kinase, an AP-2 complex subunit mu, a germin-like protein, and a hydroxyproline-rich glycoprotein (HRGP).

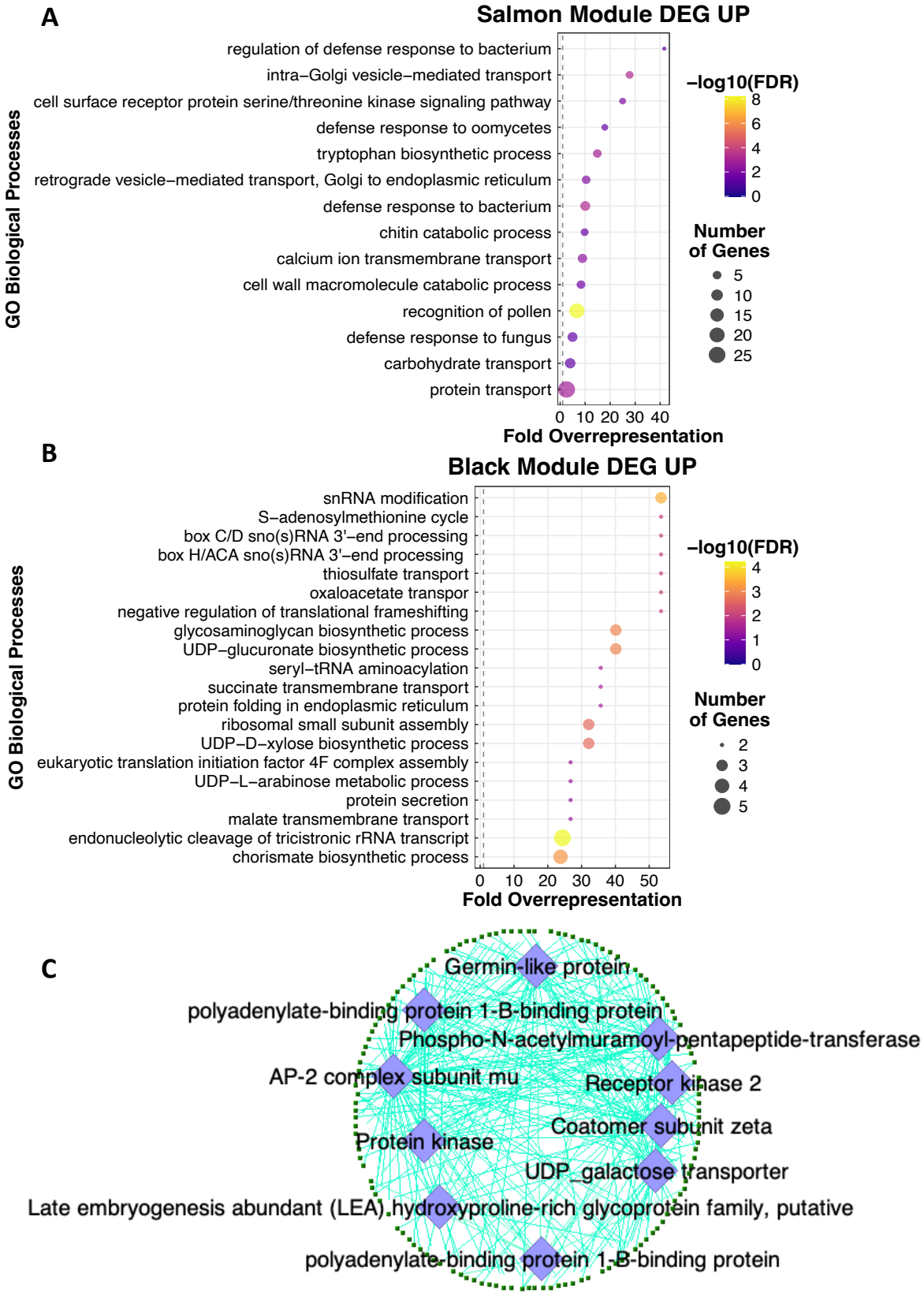
A
Salmon Module DEG UP
regulation of defense response to bacterium
intra−Golgi vesicle−mediated transport
cell surface receptor protein serine/threonine kinase signaling pathway
defense response to oomycetes
tryptophan biosynthetic process
retrograde vesicle−mediated transport, Golgi to endoplasmic reticulum
defense response to bacterium
chitin catabolic process
calcium ion transmembrane transport
cell wall macromolecule catabolic process
recognition of pollen
defense response to fungus
carbohydrate transport
protein transport
GO Biological Processes
0 10 20 30 40
Fold Overrepresentation
−log10(FDR)
8
6
4
2
0
Number of Genes
5
10
15
20
25
B
Black Module DEG UP
snRNA modification
S−adenosylmethionine cycle
box C/D sno(s)RNA 3'−end processing
box H/ACA sno(s)RNA 3'−end processing
thiosulfate transport
oxaloacetate transpor
negative regulation of translational frameshifting
glycosaminoglycan biosynthetic process
UDP−glucuronate biosynthetic process
seryl−tRNA aminoacylation
succinate transmembrane transport
protein folding in endoplasmic reticulum
ribosomal small subunit assembly
UDP−D−xylose biosynthetic process
eukaryotic translation initiation factor 4F complex assembly
UDP−L−arabinose metabolic process
protein secretion
malate transmembrane transport
endonucleolytic cleavage of tricistronic rRNA transcript
chorismate biosynthetic process
GO Biological Processes
0 10 20 30 40 50
Fold Overrepresentation
−log10(FDR)
4
3
2
1
0
Number of Genes
2
3
4
5
C
Germin-like protein
polyadenylate-binding protein 1-B-binding protein
Phospho-N-acetylmuramoyl-pentapeptide-transferase
AP-2 complex subunit mu
Receptor kinase 2
Coatomer subunit zeta
Protein kinase
UDP_galactose transporter
Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family, putative
polyadenylate-binding protein 1-B-binding protein

Fig. 4.5 A-C. Enriched Gene Ontology (GO) biological processes in upregulated DEGs from the Salmon **(A)** and Black **(B)** modules. Circle size represents the number of genes associated with each GO term, and circle color indicates the statistical significance of enrichment (-log10[FDR]), with yellow indicating higher significance. The y-axis lists GO biological processes, while the x-axis shows fold overrepresentation (observed/expected gene count). Higher fold overrepresentation reflects greater enrichment of the GO term compared to random expectation. **(C)** Co-expression network of hub genes in the Salmon module identified by WGCNA. Nodes represent genes, and edges represent co-expression relationships. Purple diamonds indicate hub genes.

Table 4.2: Hub genes ranked by highest edge weights in WGCNA modules associated with bacterial treatments (Fig. 4.1C).

GeneID	Functional annotation	Predicted Role	Reference
HORVU.MOREX.R3.5HG0522100	Germin-like protein	Oxidative stress response	Dunwell et al. 2008(Dunwell et al., 2008)
HORVU.MOREX.R3.2HG0116450	Phospho-N-acetylmuramoyl-pentapeptide-transferase	Peptidoglycan biosynthesis	Vollmer et al 2008(Vollmer et al., 2008)
HORVU.MOREX.R3.1HG0009460	polyadenylate-binding protein 1-B-binding protein	mRNA translation and stability	Gorgoni and Gray 2004(Gorgoni & Gray, 2004)
HORVU.MOREX.R3.1HG0075260	Receptor kinase 2	Response to pathogens	Goff and Ramonell 2007(Goff & Ramonell, 2007)
HORVU.MOREX.R3.6HG0605320	AP-2 complex subunit mu	Clathrin-mediated endocytosis	Fan et al 2013(Fan et al., 2013)
HORVU.MOREX.R3.1HG0073250	Coatomer subunit zeta	Vesicle-mediated intracellular transport	Kuge et al 1993(Kuge et al., 1993)
HORVU.MOREX.R3.4HG0413510	UDP_galactose transporter	Carbohydrate metabolism and cell wall biosynthesis	Norambuena et al 2002(Norambuena et al., 2002)

HORVU.MOREX.R3.4HG0408170	Protein kinase	Response to pathogens	Goff and Ramonell 2007(Goff & Ramonell, 2007)
HORVU.MOREX.R3.3HG0300120	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family, putative	Abiotic stress tolerance and cell wall integrity	Battaglia et al 2008(Battaglia et al., 2008)
HORVU.MOREX.R3.1HG0009460	polyadenylate-binding protein 1-B-binding protein	mRNA translation and stability	Gorgoni and Gray 2004(Gorgoni & Gray, 2004)

Analysis of Hormone-Mediated Defense Pathways

Based on the results of the WGCNA hub gene analysis and previous findings that indicated roles for plant hormones in RWA-bacteria-plant interactions (Luna et al., 2018), we explored defense response pathways mediated by the hormones salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA) and ethylene (ET). Thionins were also examined due to their established role as early defense responders in barley during aphid feeding (e.g., Escudero-Martinez et al. 2017).

SA Pathway Activation and Thionin Expression

The SA pathway is actively engaged in samples treated with honeydew containing higher bacterial titers. This conclusion is supported by a higher log2 fold change (L2FC) in phenylalanine ammonia-lyase (*PAL*) genes within the SA biosynthetic pathway (Fig. 4.6A). Four of six *PAL* genes were significantly upregulated over time (indicated by black stars), and one *PAL* gene was identified as a hub gene in the WGCNA Salmon module (boxed).

Nonexpressor of Pathogenesis-Related Genes 1 (*NPR1*), a key regulator of SA signaling, is upregulated, indicating strong SA-mediated signaling activity (Fig. 4.6A). Similar to *PAL*, the activation of *NPR1* increased over time, further supporting the sustained engagement of the SA signaling pathway. A prominent trend observed in the SA pathway is the significant upregulation of Pathogenesis Related (*PR*) genes over time, a well-established marker of SA-mediated defense. Nine of 16 *PR* genes were significantly upregulated at one more time point (Fig. 4.6A).

SA biosynthesis, signaling and response show increased gene expression levels over the 72 h time course, with the highest expression observed at 48 h in co-fed compared to isolated treatments (Fig. 4.6A). These findings highlight an enhanced SA-mediated defense response triggered by the presence of honeydew-associated bacteria.

Although not always classified as PR genes (Terras et al., 1993), thionins share functional similarities with PR proteins and are relevant in SA-regulated plant defense responses (Sharma et al., 2021). Expression of thionin genes was significantly increased over time in co-fed vs. isolated treatments in both plants treated with honeydew and those fed on by RWA (Fig. 4.6B).

Given that thionins are induced by microbial signals, and that thionin gene expression was high in plants exposed to bacteria-rich honeydew and RWA feeding, we wanted to confirm that bacteria were the source driving thionin expression. Using qRT-PCR, we analyzed thionin expression in barley plants infiltrated with honeydew from co-fed aphids or *Winslowiella iniecta*, a bacterium that was cultured from honeydew collected from co-fed aphids and absent in honeydew collected from isolated aphids (Fig. 4.6C). The thionin expression patterns were similar in plants infiltrated with honeydew from co-fed aphids and *W. iniecta*, consistent with a role of for bacteria in driving thionin expression (Fig. 4.6D).

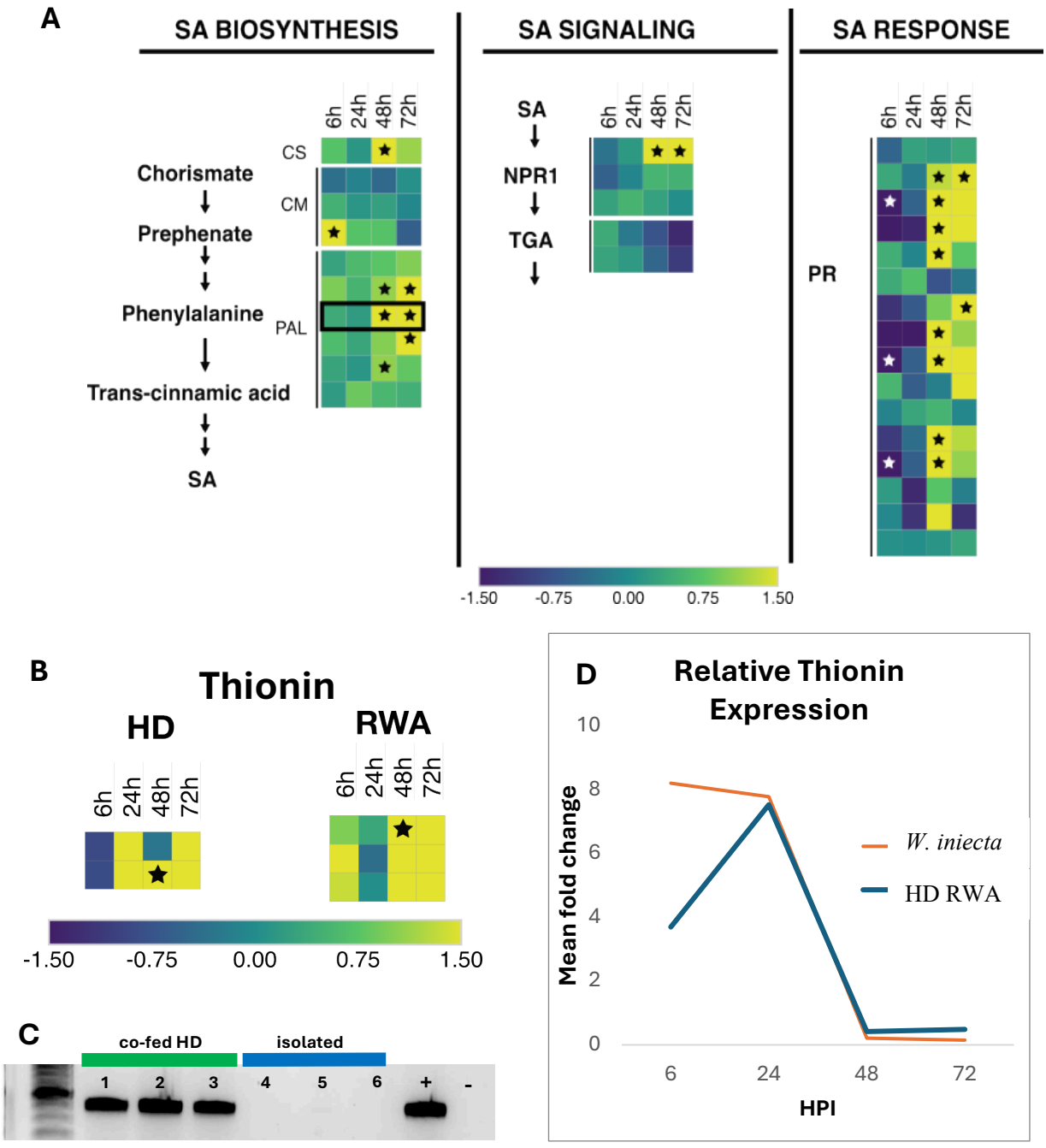

Fig. 4.6. (A) Expression patterns in the salicylic acid (SA) biosynthesis and signaling pathways in barley plants infiltrated with honeydew collected from co-fed and isolated aphids. **(B)** Thionin gene expression patterns in RNA-Seq experiments comparing co-fed vs. isolated honeydew infiltration and co-fed vs. isolated RWA feeding. For all heatmaps, colors indicate log2 fold changes (co-fed compared to isolated), ranging from purple (low expression) to yellow (high expression). Black stars denote significantly upregulated DEGs, white stars indicate significantly downregulated DEGs, and boxed genes represent hub genes in the WGCNA Salmon module. **(C)**

Gel electrophoresis image showing amplification of DNA specific to *Winslowiella iniecta*. Honeydew DNA from samples 1-3 was collected from co-fed honeydew enrichments and samples 4-6 was collected from isolated honeydew enrichments. The '+' represents the positive control (*W. iniecta* B120) while the '-' represents negative control (water). **(D)** Mean fold change of thionin expression in barley at 6, 24, 48, 72 h post infiltration of *W. iniecta* or feeding on co-fed aphids as assessed by qRT-PCR.

Genes in the JA Biosynthetic Pathway are Active, But JA Signaling is Repressed

Several key genes in the JA biosynthetic pathway were differentially upregulated in co-fed honeydew treatments, including three lipoxygenase (*LOX*) genes, one allene oxide cyclase (*AOC*) gene, and two 12-oxo-phytodienoic acid (*OPDA*) genes (Fig. 4.7), suggesting JA synthesis is activated by honeydew associated microbes. Notably, one of the upregulated LOX genes encodes LOX2.1, a chloroplast-localized enzyme with confirmed 13-lipoxegenase activity, the first committed step in JA biosynthesis (UniProtKB -P93184). The *CORONATINE INSENSITIVE 1* (*COI1*) receptor, which is essential for JA perception and activation of JA-dependent responses, showed a slight downregulation over time in plants treated with honeydew containing a high microbial load, suggesting reduced JA perception at later timepoints. However, it is important to note that *COI1* expression is active in both treatments, as shown by comparison with water infiltrated leaves.

Data from this study suggest that the defense responses resulting from JA signaling are suppressed by microbes associated with honeydew (Fig. 4.7). First, genes encoding JASMONATE ZIM-Domain (*JAZ*) proteins, key repressors of JA signaling, were significantly upregulated following treatment with honeydew from co-fed aphids. In fact, one *JAZ* gene was identified as a hub gene (Fig. 4.7, Supplementary Table S4.1), suggesting that JA signaling is reduced in

response to microbes in the honeydew. In addition, JA-induced oxygenases (JOX), which degrade JA, were upregulated at early timepoints but showed reduced expression at later timepoints, suggesting that while JA is initially produced, it is rapidly degraded before full repression (via JAZ upregulation) is established. Although genes encoding NOVEL INTERACTOR OF JAZ (*NINJA*), a co-repressor of JA signaling, were not significantly differentially expressed, they showed a slight upregulation at early timepoints, potentially contributing to early-stage repression of the signaling pathway. In contrast, genes encoding MYC-Related Transcription Factor 2 (*MYC2*), the transcription factor required for JA-induced responses, were not differentially expressed. Further confirmation of JA signaling repression is provided by the downregulation of a JA-induced protein.

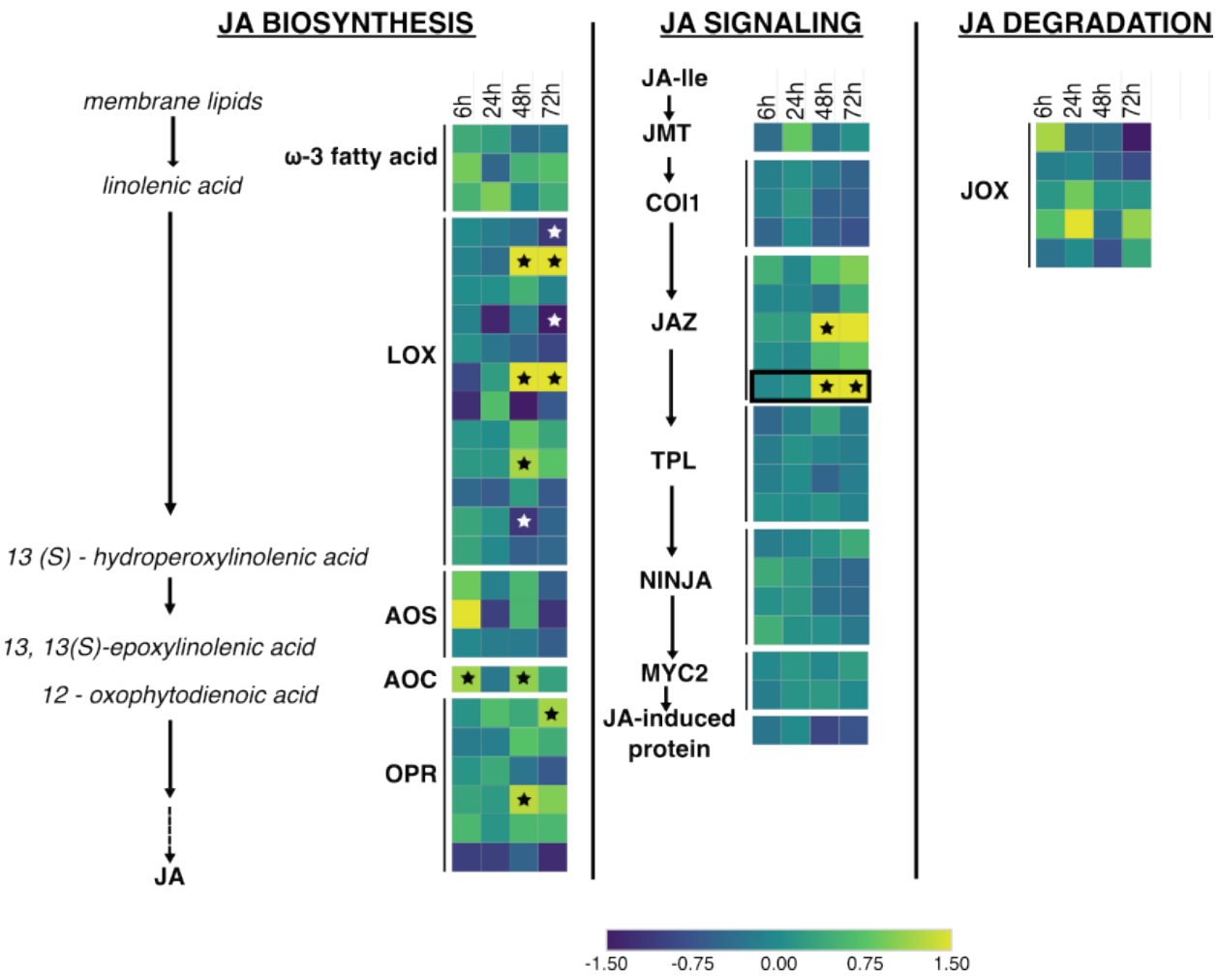

Fig. 4.7. Expression patterns in the jasmonic acid (JA) biosynthesis and signaling pathways in barley plants infiltrated with honeydew collected from plants treated with co-fed or isolated aphids. Colors indicated log2 fold changes (co-fed compared to isolated), ranging from purple (low expression) to yellow (high expression). Black stars denote upregulated DEGs, white stars indicate downregulated DEGs, and boxed genes represent hub genes in the WGCNA Salmon module.

Hormonal Cross-Talk in Stress Responses

Given that genes related to ABA and ET were identified in the DEG and hub gene analyses, we looked at their regulatory pathways for potential involvement in the plant's

response to microbial rich honeydew. Biosynthesis, perception and signaling pathway genes were not differentially upregulated. However, two ethylene response factor (*ERF*) genes were identified as hub genes, and 11 *ERF* genes were significantly upregulated (Supplementary Fig. S4.5).

Likewise, there was no strong evidence for involvement of the ABA pathway in responses to the honeydew rich in bacteria. ABA biosynthesis genes were minimally induced, and the signaling pathway is only weakly engaged. However, ABA-responsive genes were induced in co-fed relative to isolated honeydew treatments, with 16 upregulated genes, including Late embryogenesis abundant protein genes (*LEA*) and GRAM domain-containing proteins (*GRAM*) (Supplementary Fig. S4.6).

4.4 DISCUSSION:

This study demonstrates that RWA honeydew serves as a medium for microbial transmission and that honeydew-associated microbes actively modulate plant defense responses. Our results reveal a strong differential regulation of the JA and SA pathways, with the JA pathway being actively suppressed by *JAZ* repressors and oxidative processes, while the SA pathway is highly induced, favoring a defense strategy that may facilitate aphid feeding. This shift in signaling is reinforced by our co-expression network analysis, which identified key regulators of JA suppression, SA activation and potential hormonal cross-talk.

Microbial Transmission and Honeydew Microbiome

Our results provide direct evidence that microbes in honeydew --not just aphid feeding-- can drive changes in plant defense signaling. Unlike other hemipteran systems where microbes

reside inside the insect's mouthparts and are directly injected into the plant via the stylet (e.g., *Xylella fastidiosa* in the blue-green sharpshooter (Newman et al., 2004)), our SEM and TEM studies detected microbes on the aphid exterior but not internally, suggesting a different mode of microbial transmission.

We propose a model in which aphids acquire microbes from honeydew-contaminated leaf surfaces as they probe, introducing them into plant tissues via stylet contamination (Fig. 4.10A). As the stylet punctures or passes cells, it deposits gelling saliva, microbes and microbial effectors, which may interact with host receptors to alter defense signaling (Fig. 4.10B).

The microbial composition of honeydew mirrors many components of the RWA microbiome (Luna et al., 2018), but notably contains very few OTUs mapping to *Buchnera aphidicola,* the obligate symbiont. This suggests that the RWA-associated microbes are derived either from gut symbionts, co-feeding or environmental acquisition. Honeydew from isolated aphids harbored significantly fewer microbes, further supporting that microbial transmission depends on aphid density and feeding interactions.

Given that honeydew is rich in sugars and amino acids, it provides an optimal environment for microbial colonization (Lanan, 2020; Leroy et al., 2011).

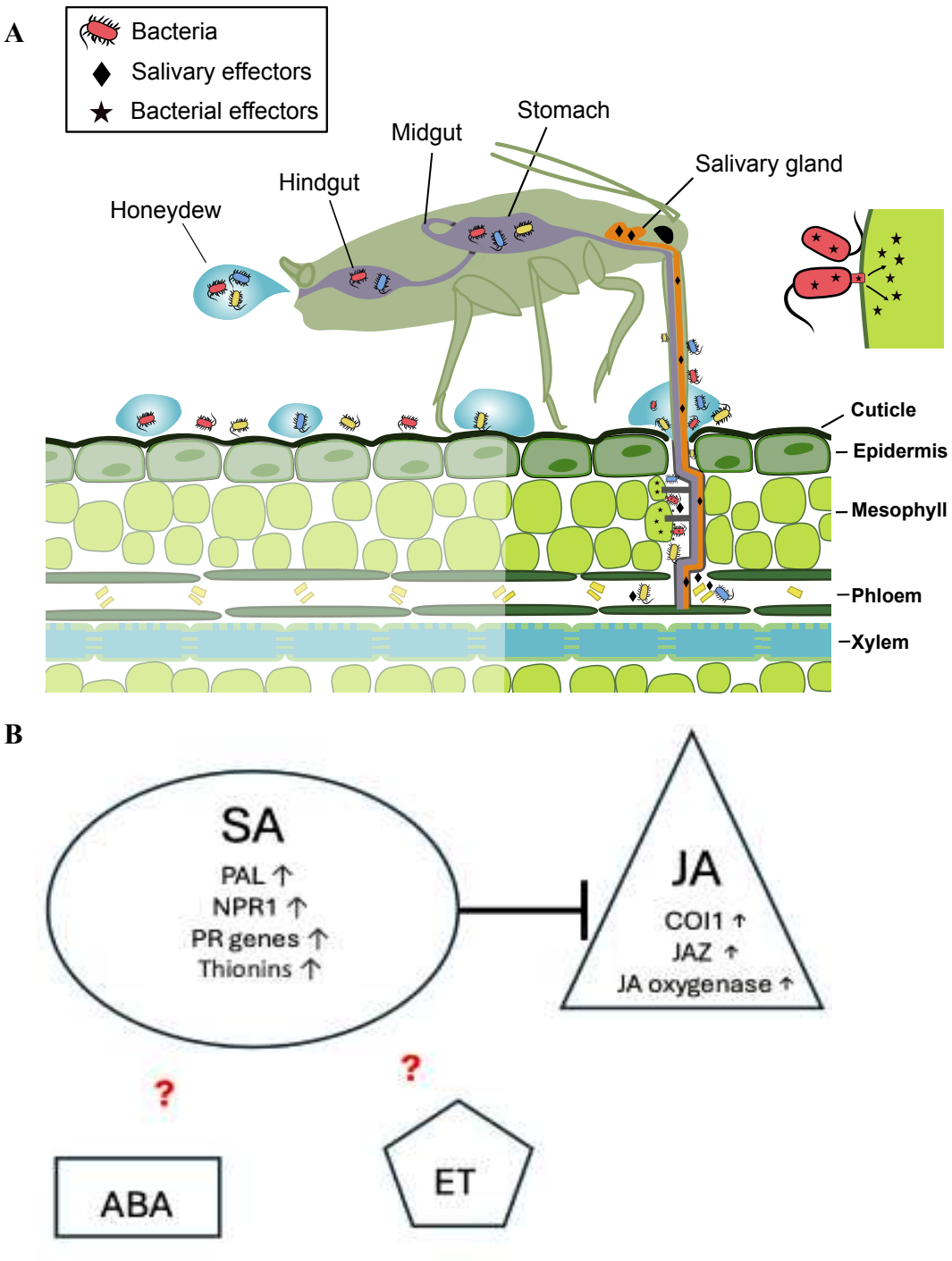

Fig. 4.10 A & B: (A) Schematic of Russian wheat aphid (RWA) feeding on leaf contaminated with honeydew. As the stylet punctures the leaf surface it carries microbes and/or microbial effectors from the honeydew into the plant tissue. The stylet moves extracellularly but samples cells by puncturing them. During this process, it deposits gelling saliva along with microbes and/or microbial effectors from the stylet surface into the cell cytoplasm. These along with

putative aphid effectors trigger plant defense responses. **(B)** Key plant defense responses induced by honeydew microbes. Transcriptome profiles of barley plants treated with honeydew from co-fed RWA vs honeydew from isolated aphids showed upregulation of SA-biosynthetic and downstream-regulated genes. Although JA biosynthetic genes were upregulated, the concurrent upregulation of genes involved in JA suppression (*coi1* and *jaz*) and JA degradation (*jox*) suggest reduced JA accumulation.

Regulation of Plant Defense Hormones in Honeydew-Microbe-Plant Interactions

Our transcriptome analysis reveals a time-dependent response to RWA-associated microbes, introduced through either honeydew infiltration or aphid feeding. Additionally, qPCR analysis shows upregulation of the plant protective protein thionin by the RWA-associated microbe *W. iniecta.* The common upregulation of thionin by cofed RWA, honeydew from cofed aphids, and *W. iniecta* further supports microbial involvement in modulating plant defenses.

Microbial Influence on JA Biosynthesis and Repression

JA signaling is tightly regulated by interactions between COI1 (JA-Ile receptor) and JAZ (repressor). Under normal conditions, when JA-Ile accumulates, it binds to the COI1 receptor, leading to ubiquitin-mediated degradation of JAZ proteins. This degradation releases MYC2 from JAZ-mediated repression, allowing it to activate JA-induced defense genes (Gupta et al., 2020). Our results indicate a disruption in this classical JA signaling cascade.

Despite upregulation of JA biosynthetic genes (*LOX*, *AOS*, *ODPA*), *COI1* expression was downregulated over time, suggesting reduced JA perception in plants treated with honeydew containing high microbial loads. *JAZ* genes, key repressors of JA signaling, were significantly upregulated, with one *JAZ* gene identified as a hub gene, reinforcing that JA signaling is actively

repressed in response to honeydew-associated microbes. This suggests a decoupling of JA biosynthesis from its downstream signaling, potentially allowing for microbes associated with aphids to dampen JA-mediated defenses while still maintaining some biosynthetic activity.

Interestingly, JA-induced oxygenases (JOX), which degrade JA, were upregulated at early timepoints. This suggests that although JA is initially produced, it is rapidly degraded before JAZ-mediated repression is established. Given that JA degradation occurs in the endoplasmic reticulum (Gupta et al., 2020), we postulate that JA degradation is occurring before repression by JAZ becomes dominant.

Ethylene Response Factor (ERF) and Hormonal Cross-Talk

Two hub genes and 11 DEGs encoding ERFs were upregulated, indicating that these transcription factors play a role in the plant's response to aphid-associated microbes. However, their mode of activation remains unclear. Classically, ERFs are activated via ET or JA (Binder, 2020). JA-signaling branches into two distinct pathways: the MYC2-dependent pathway, which regulates wounding- and insect-induced JA responses and the ERF-dependent pathway, which regulates necrotrophic-induced JA responses (Gupta et al., 2020). ERFs are known to interact with JAZ proteins and mediate cross-talk between JA and other hormonal pathways, including ET and SA (Sui et al., 2025).

In the co-fed honeydew treated plants, JA suppression coincides with high ERF expression, suggesting an alternative regulatory mechanism. Given the strong induction of SA, it is possible that SA is involved in driving ERF activation, further reinforcing JA suppression and

shifting the plant's defense away from insect resistance. However, further investigation is needed to experimentally confirm this hypothesis.

SA as a Dominant Defense Pathway

The SA biosynthetic and downstream pathways were highly induced, reinforcing the idea that SA mediated defenses are triggered in response to microbial presence. This, in combination with the apparent inhibition of JA accumulation, suggests SA dominates the plant's defense response.

Hub Genes and Hormonal Regulation in Plant Defense

Several hub genes identified in our analysis may contribute to the suppression of JA signaling and the broader regulation of plant defenses in response to honeydew-associated microbes. Notably, we identified a JAZ gene as hub gene, supporting that repression of JA signaling plays a key role in aphid-microbe-plant interactions. The identification of two ethylene-responsive transcription factors (ERFs) as hub genes suggests a potential cross-talk between JA, SA and ET pathways. Given that ERFs are typically activated via JA or ET but no induction of ET biosynthesis was observed in our dataset, their upregulation may be occurring through alternative signaling pathways, possibly influenced by SA.

Additionally, our analysis identified phenylalanine ammonia-lyase (PAL), a key enzyme in SA biosynthesis, as a hub gene, reinforcing the strong SA activation observed in plants treated with microbe-enriched honeydew. The presence of an ABA-responsive protein as a hub gene suggests that ABA may play a minor role, although ABA biosynthesis genes were not strongly induced.

As 44 protein kinases, including a receptor kinase, were identified as hub genes, kinase signaling must play a large role in shaping responses to honeydew associated microbes. Protein kinases are known regulators of hormone signaling, particularly in JA and SA-mediated responses (Goff & Ramonell, 2007).

One intriguing finding is the identification of the *MraY* gene (HORVU.MOREX.R3.2HG0116450) as a hub gene. *MraY* encodes phospho-N-acetylmuramoyl-pentapeptide-transferase, an enzyme in peptidoglycan biosynthesis, a process primarily associated with bacterial cell walls (Ikeda et al., 1991). The presence of this gene in barley raises questions about its functional role. One possibility is that it represents a remnant of conserved pathways between chloroplasts and bacteria, as peptidoglycan remnants have been observed around chloroplasts in some early diverging plant lineages (Dowson et al., 2022). Alternatively, plants may have evolved to mimic certain bacterial-like features, potentially influencing microbial interactions. Although its relevance in barley remains unclear, the identification of this gene alongside multiple defense-related hub genes suggests it may be involved in plant-microbe interactions.

Our results support a model in which honeydew-associated microbes regulate JA signaling to facilitate aphid feeding. While JA biosynthesis is induced, multiple layers of suppression, including JAZ accumulation, COI1 regulation, and JA degradation, effectively prevent a strong JA-mediated defense response. The strong upregulation of SA-related genes, including PAL, further suggests that SA signaling dominates in response to bacterial-rich honeydew, and is consistent with JA suppression.

Understanding how RWA-associated microbes influence plant defense responses may offer new pest control strategies. The repression/suppression of JA signaling by honeydew-associated bacteria suggests a potential target for disrupting aphid-plant interactions. Beyond pest control, these findings have broader implications for plant-microbe interactions and crop protection. The ability of RWA-associated microbes to modulate host defenses raises questions about the role of similar microbial interactions in other agricultural pests. How these communities influence plant immunity across different systems could improve disease resistance strategies.

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Chapter 5: CONCLUSION:

Conclusions and Future Directions

Food security is a global crisis, and the need for sustainable production of staple crops like wheat requires innovative approaches for insect management strategies. RWA infestations have caused significant agricultural losses worldwide and remain a major threat to wheat and barley production. Chemical control is often ineffective, as aphids can evade treatment by hiding in curled leaves. Moreover, only a few effective *R* genes remain available to breeders, and it's only matter of time before aphids overcome this resistance. This highlights the urgent need for alternative control strategies. This research provides key insights into how aphid-associated microbial communities influence aphid virulence and modulate plant defenses, opening new avenues for pest management.

One of the most striking findings from this work is that the aphid salivary proteome was comprised mainly of bacterial peptides and the same genera of bacteria are associated with RWA. Populations of aphids with higher titers of bacteria caused more chlorosis to wheat plants (Luna et al., 2018). Another surprising finding was that some microbes, such as *W. inieca*, were found in populations of aphids with higher bacterial titers, and this microbe was previously unknown to science (Campillo et al., 2015; Luna et al., 2018). The genome of *W. iniecta* is particularly interesting, as it contains putative secretion systems associated with both animal and plant interactions, highlighting the bacterium's flexibility in interacting with both aphids and their host plants (Campillo et al., 2015). Next, we found that this microbe, along with others, is present in aphid honeydew, and that bacterial-enriched honeydew elicits plant

responses similar to those triggered by bacteria-enriched aphids. This highlights the role of bacteria in suppressing plant defenses, thereby facilitating aphid feeding and colonization. The induction of plant defenses is primarily associated with an SA response, while JA is downregulated, likely through JAZ repressors or JA oxidase activity.

Future directions for this project include:

1. **Linking microbial function to aphid virulence.** This could be achieved through comprehensive genome analysis of sequenced strains to identify genes or pathways predicted to contribute to bacterial virulence and/or the induction of plant defense responses. For example, *W. iniecta* contains two predicted type III effectors: a *Salmonella*-like effector (SseCB) and an *Erwinia*-like effector (DspEF) (Campillo et al. 2015). The *Salmonella*-like effector interacts with animal hosts, while the *Erwinia*-like effector interacts with plants, suggesting that *W. iniecta* can engage with both the aphids and plants. The roles of these effectors could be further studied through mutant analysis.

   A means to determine which microbes specifically contribute to aphid virulence could be studied by inoculation of plants with SynComs (synthetic microbial communities) (Großkopf & Soyer, 2014). SynCom construction would be guided by several factors, including amplicon sequencing, metagenomic sequencing and metatransciptomic sequencing (Marynowska et al., 2017) of aphid microbiomes, which would help link microbial functions to RWA virulence.

Another approach to validate microbial contributions would be to investigate the effects of microbiome alterations in isolated aphids. This could be achieved by allowing co-fed aphids to feed on artificial diet for several days, then replacing them with isolated aphids, effectively transferring their microbiomes. Alternatively, leaves could be treated with honeydew or bacteria isolated from co-fed aphids, followed by isolated aphid feeding assays to determine whether isolated aphids become more virulent after exposure to the co-fed honeydew infiltrates or specific bacterial isolates.

2. **Elucidating how the bacteria are delivered to the interior of the plant.** To confirm that bacteria present in honeydew are introduced into the plant via aphid feeding, we could use a labeling approach with red fluorescent protein (RFP)--labeled bacteria, allowing us to track their movement through different stages. For this experiment, we would first monitor the bacterial uptake by tracing labeled bacteria through the aphid's gut and then determine whether labeled bacteria are secreted in honeydew, confirming their survival through the digestive tract. Next, we would examine aphid stylets for the presence of labeled bacteria to assess their potential transmission during feeding. Finally, we would assess whether bacteria from honeydew are delivered into plant tissues via aphid stylet penetration. To visualize bacterial movement, florescence microscopy and confocal imaging would be used at each step. Additionally, quantitative PCR or plating assays could be performed to measure bacterial titers at different stages of transmission. This approach would provide critical evidence for how aphid-associated microbes interact with both the insect vector and the plant host.

3. **Study the implications of the suppression of the JA pathway**. The suppression of the JA pathway could be explored by qRT-PCR analysis of additional JA biosynthesis and signaling genes, as well as downstream targets like *VSP1* (Vegetative Storage Protein 1) and *PDF1.2* (Plant Defensin 1.2). Several biological experiments could further explore these interactions, including treating plants with SA to assess effects on aphid feeding, conducting hormone analysis on barley leaves following honeydew infiltration.

    To further dissect the contributions of specific hormones, wheat or barley mutants deficient in SA or JA signaling could be used to evaluate the functional implications of pathway suppression. This would be assessed by introducing a specific number of aphids to plants, allowing them to feed for a set period, and then measuring aphid fitness. Fitness metrics could include reproduction and development (fecundity, nymphal development time, adult longevity), population growth, feeding behavior (electrical penetration graph analysis, honeydew production) and plant damage and stress indicators (chlorosis and leaf rolling).

Discoveries from this work will increase our understanding insect-microbe-plant interactions and reveal key points for improving pest control. For example, *should resistance breeding efforts target microbes that enhance virulence?* or *could treatments that modify RWA or wheat microbiomes provide protection against infestations?* Addressing these questions will be crucial for developing sustainable strategies to safeguard wheat production and global food security.

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Appendix A

Supplementary Materials

A.1 Supplementary Materials Chapter 2: BACTERIA ASSOCIATED WITH RUSSIAN WHEAT APHID (*DIURAPHIS NOXIA*) ENHANCE APHID VIRULENCE TO WHEAT

Supplementary Table S2.1. Species composition of the mock community for 16S rRNA sequencing.

Bacteria	Strain	Host	Reference
Erwinia iniecta	B120	Artificial diet fed on by RWA	(Campillo et al., 2015)
Putative *Erwinia-Enterobacter sp.*	B116	RWA whole aphid	This study
Putative *Staphylococcus* sp.	N/A	Salivary gland of whole aphid	This study
Putative *Paenibacillus* sp.	B142	RWA whole aphid	This study
Putative *Arthrobacter* sp.	B145	Artificial diet fed on by RWA	This study
Putative *Enterobacter* sp.	B156	Artificial diet fed on by RWA	This study
Putative *Pantoea*	B151	Artificial diet fed on by RWA	This study
Xanthomonas oryzae pv. *oryzae*	MAFF311018	*Oryza sativa*	(Ochiai et al., 2005)
Xanthomonas euvesicatoria	85-10	*Capsicum*	(Thieme et al., 2005)

Lonsdalea quercina	NCCB100490	*Quercus rubra*	(Caballero et al., 2014)
Escherichia coli	DH5-alpha		cgsc2.biology.yale.edu
Burkholderia andropogonis	3549	*Saccharum officinarum*	(Zhao et al., 2004)

Supplementary Table S2.2. iTRAQ comparison of secreted salivary proteins of *D. noxia* biotypes 1 and 2.

Accession number	Protein homology	e-value[a]	Sequences[b]	Fold-change ratio biotype 2/biotype 1[c]		
				Rep 1	Rep 2	Rep 3
gi|193676365	ACYPI000986, *glucose dehydrogenase*	1.2e-05	20(3)	0.362	0.331	0.663
gi|328709186	ACYPI000113, *glucose dehydrogenase*	1.3e-02	4(4)	0.395	0.503	0.648
gi|193659536	ACYPI000288, *glucose dehydrogenase*	2.5e-04	22(3)	0.343	0.556	0.445
gi|641667063	ACYPI000422, *apolipophorin*	8.7e-03	9(2)	0.249	0.576	0.407
gi|328719823	ACYPI006675, *aminopeptidase N*	0.057	1(1)	0.195	0.257	0.229
gi|328713749	ACYPI25151, *mucin-17-like*	0.05	1(1)	0.274	-	0.689
gi|641679172	LOC100574293, *DDB_G0282133*	0.01	1(1)	0.172	0.220	0.575
gi|641662958	LOC100169225, *aminopeptidase N-like*	0.081	1(1)	0.225	0.256	0.228
gi|193580006	ACYPI009881, *putative sheath protein*	0.078	2(1)	0.414	0.503	0.615
gi|193659688	ACYPI003908, *adenosylhomocysteinase*	0.052	1(1)	-	-	-
gi|328705553	ACYPI008945, *titin-like*	0.2	1(1)	0.183	0.428	0.333
gi|193715980	ACYPI002298, *trehalase-like*	0.3	1(1)	0.211	0.487	0.584
gi|239789413	ACYPI23752, *carbonic anhydrase*	0.22	1(1)	0.628	0.326	0.529

[a]Expected value for the highest-scoring peptide contributing to protein identification.

[b]Total number of unique peptides contributing to the score, with number of unique peptides with iTRAQ ratios contributing to quantification in parenthesis.

[c]Weighted ratios. For each reporter ion, the intensity values of the assigned peptides are summed and the protein ratios calculated from the summed values. Ratios in bold are significantly different from 1 at a 95% confidence level. Data were normalized using summed intensities. A correction factor was applied such that the sum of the intensities for a reporter ion peak over all peptide matches that pass the quality tests is the same for all the replicates. Adenosylhomocysteinase, titan-like, trehalase-like and carbonic anhydrase-like were included although their high scoring peptide had an E-value greater than 0.05.

Supplementary Table S2.3. Turbidity (OD_{600nm}) of artificial diets after 24 and 48 hours of aphid feeding.

Treatment	OD600 ± SD
No aphids 24 h	0.006 ±0.008
No aphids 48 h	0.003 ± 0.003
Isolated aphids 24 h	0.025 ± 0.009
Isolated aphids 48 h	0.003 ± 0.008
Control aphids 24 h	0.013 ± 0.003
Control aphids 48 h	0.003 ± 0.001

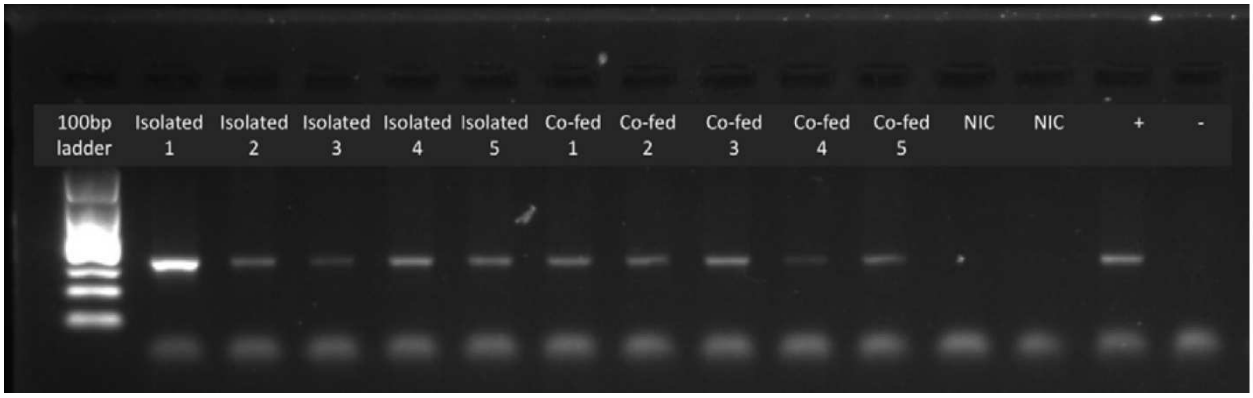
Supplementary Table S2.4. Accession numbers and size of 16S rRNA sequences used to construct the phylogenetic tree in Fig. 2.2. Type strains are indicated by a superscript T.

Genus/Species	Strain/isolate	Accession number	Size (nt)	*Reference*	*Source*
Type strains:					
Acinetobacter antiviralis	KNF2022	DQ314740	1445		
Acinetobacter baumanii	JCM 6841^{T}	AB594765	1459		
Acinetobacter soli	B1^{T}	EU290155	1422		
Enterobacter asburiae	LMG 26064^{T}	AB004744	1422		
Enterobacter aerogenes	LMG 2094^{T}	AB004750	1438		
Pantoea agglomerans	LMG1286^{T}	AB681812	1466		
Pantoea eucalypti	LMG 24197^{T}	EF688009	1348		
Pantoea vagans	LMG 24199^{T}	EF688012	1429		
Erwinia toletana	LMG 24162^{T}	AF130964	1475	(Rojas et al., 2004)	
Erwinia amylovora	CFBP 1232^{T}	Z96088	1481		
Erwinia aphidicola	CFBP 6829^{T}	AB681773	1461		
Buchnera aphidicola	M63249				
D.noxia/saliva/wheat strains					
Putative *Acinetobacter* sp.	B114	KP996668	1459	This study	*Artificial diet fed on by RWA*

Putative *Erwinia-Enterobacter* sp.	B116	KP064323	1539	This study	*Wheat leaf fed on by RWA*
Putative *Erwinia-Enterobacter* sp.	B119	KP064324	1300	This study	*RWA whole aphid*
Erwinia iniecta	B120^{T}	KM870781	1495	(Campillo et al., 2015)	*Artificial diet fed on by RWA*
Putative *Erwinia-Enterobacter* sp.	B147	KP064325	1417	This study	*Artificial diet fed on by RWA*
Putative *Erwinia-Enterobacter* sp.	B148	KP064326	1418	This study	*Artificial diet fed on by RWA*
Putative *Pantoea* sp.	B151	KR003365	900	This study	*Artificial diet fed on by RWA*
Putative *Pantoea* sp.	B152	KR003366	729	This study	*Artificial diet fed on by RWA*
Putative *Enterobacter* sp.	B156	KR003367	882	This study	*Artificial diet fed on by RWA*

Supplementary Table S2.5. Wheat Affymetrix GeneChip probe set IDs with homology to genes in the jasmonic acid (JA) or salicylic acid (SA) biosynthetic pathways, and with calculated log2 fold changes in excess of 1.000 at 5 or 48 h post-infestation with *D. noxia* biotype 1, as compared to uninfested controls (*n* = 3 per sample type)

Pathway	Description	Probe ID	5 h post-infestation			Uninfested control			Log_2 FC	48 h post-infestation			Uninfested control			Log_2 FC
JA	omega-3 fatty acid desaturase	Ta.701	6.625	6.278	6.705	7.712	7.813	8.418	-1.445	8.884	8.408	8.926	10.249	10.028	10.141	-1.400
	lipoxygenase	Ta.24254	9.124	9.473	9.663	4.664	4.096	3.829	5.224	6.639	7.226	6.889	7.494	7.364	7.901	-0.668
		Ta.12757	9.147	8.927	9.099	5.836	5.866	6.347	3.042	8.200	8.693	8.235	8.184	7.924	8.969	0.017
		Ta.13650	11.058	10.992	11.233	8.977	8.302	8.528	2.492	9.967	9.920	10.523	7.486	7.954	7.881	2.363
		Ta.28171	11.979	11.962	11.917	4.190	4.438	4.242	7.662	6.221	7.967	7.526	6.664	6.825	6.376	0.616
		Ta.22828	10.170	9.796	8.801	3.619	3.438	3.619	6.031	5.030	5.179	5.086	9.174	7.611	5.199	-2.230
		Ta.9742	9.677	9.417	9.608	7.027	7.138	7.288	2.417	8.553	8.901	8.765	8.868	8.493	8.854	0.001
		Ta.526	8.644	8.220	6.889	3.006	2.865	2.584	5.099	4.751	4.875	4.668	6.280	5.746	4.133	-0.622
	allene oxide synthase	TaAffx.80134	7.265	7.593	7.733	5.390	5.236	5.231	2.244	6.924	7.067	6.620	6.564	6.737	7.132	0.059
		TaAffx90316	8.302	9.358	9.485	8.254	5.932	6.024	2.312	4.979	5.403	5.522	5.760	6.674	6.278	-0.936
		Ta18134	4.321	3.936	3.808	3.324	3.650	3.185	0.636	4.398	4.460	4.396	7.500	5.206	4.266	-1.240
		Ta18630	6.709	6.786	6.728	4.579	4.289	5.109	2.082	5.922	6.228	6.078	6.456	6.413	6.258	-0.299
	allene oxide cyclase	Ta27217	12.900	12.726	12.721	6.174	5.729	5.934	6.837	6.896	7.949	7.350	8.913	8.792	7.408	-0.973
	12-oxo-PDA reductase	Ta7703	9.555	10.252	10.259	6.296	6.205	5.862	3.901	7.081	7.045	7.056	8.571	8.804	8.347	-1.513
		Ta1207	7.087	7.207	7.708	3.932	4.124	4.375	3.191	7.081	7.045	7.056	8.571	8.804	8.347	-1.513
		Ta27016	7.130	5.727	6.272	8.350	8.362	7.988	-1.857	9.312	9.935	9.718	7.624	7.586	8.785	1.657
		Ta30735	4.741	3.081	3.434	2.605	2.708	2.477	1.155	5.025	5.041	4.425	5.530	5.207	5.624	-0.623
		Ta5509	9.679	9.245	9.271	7.627	7.403	7.565	1.866	8.301	8.459	8.432	8.675	8.503	9.374	-0.453
SA	prephenate dehydratase	Ta9122	8.204	8.330	8.345	6.287	6.044	5.885	2.221	7.962	8.353	8.279	7.669	7.433	7.917	0.525
	phenylalanine ammonia lyase	Ta7022	7.992	7.271	7.358	3.804	3.854	3.259	3.901	6.650	7.410	6.764	6.419	6.578	6.832	0.332
		Ta9220	9.648	9.603	9.770	3.007	2.725	2.508	6.927	4.019	7.980	5.223	6.025	4.585	5.338	0.425
	4-coumarate: CoA ligase	Ta5623	10.197	10.281	10.292	6.909	6.637	6.528	3.565	9.996	9.994	9.889	10.610	10.593	10.477	-0.600
		Ta30476	5.789	5.668	6.002	4.505	4.666	4.408	1.293	5.505	5.668	5.389	5.584	5.380	6.162	-0.188
	PR-1	Ta62	6.880	4.825	4.478	4.193	4.581	3.975	1.144	9.300	12.547	10.449	8.750	9.673	10.832	1.014



Supplementary Fig. S2.1. Gel electrophoresis visualization of PCR using V4 region of the 16S rRNA (primers 515F/806R) of DNA isolated from aphids for amplicon sequencing. Samples labeled 'Isolated' or 'Co-fed' are aphid DNAs and samples labeled 'NIC' are negative isolation controls; '+' is positive amplification control (*E. iniecta*) and '-' is negative amplification control (sterile distilled water).

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                                  20                  40                  60                  80
Erwinia iniecta B120  TACGGAGGGT GCAAGCGTTA ATCGGAATTA CTGGGCGTAA AGCGCACGCA GGCGGTTTGT TAAGTCAGAT GTGAAATCCC 80
Enterobacteriaceae sp.TACGGAGGGT GCAAGCGTTA ATCGGAATTA CTGGGCGTAA AGCGCACGCA GGCGGTTTGT TAAGTCAGAT GTGAAATCCC 80
                                 100                 120                 140
Erwinia iniecta B120  CGGGCTCAAC CTGGGAACTG CATTTGAAAC TGGCAAGCTT GAGTCTCGTA GAGGGGGGTA GAATTCCAGG 150
Enterobacteriaceae sp.CGGGCTCAAC CTGGGAACTG CATTTGAAAC TGGCAAGCTT GAGTCTCGTA GAGGGGGGTA GAATTCCAGG 150

                                  20                  40                  60                  80
Erwinia sp.  B147     TACGGAGGGT GCAAGCGTTA ATCGGAATTA CTGGGCGTAA AGCGCACGCA GGCGGTCTGT CAAGTCAGAT GTGAAATCCC 80
                      TACGGAGGGT GCAAGCGTTA ATCGGAATTA CTGGGCGTAA AGCGCACGCA GGCGGTCTGT CAAGTCAGAT GTGAAATCCC 80
                                 100                 120                 140
Erwinia sp.  B147     CGGGCTTAAC CTGGGAACTG CATTTGAAAC TGGCAGGCTA GAGTCTTGTA GAGGGGGGTA GAATTCCAGG 150
                      CGGGCTTAAC CTGGGAACTG CATTTGAAAC TGGCAGGCTA GAGTCTTGTA GAGGGGGGTA GAATTCCAGG 150

                                  20                  40                  60                  80
Enterobacteriacae sp.TACGGAGGGT GCAAGCGTTA ATCGGAATTA CTGGGCGTAA AGCGCACGCA GGCGGTCTGT CAAGTCGGAT GTGAAATCCC 80
B156                  TACGGAGGGT GCAAGCGTTA ATCGGAATTA CTGGGCGTAA AGCGCACGCA GGCGGTCTGT CAAGTCGGAT GTGAAATCCC 80
                                 100                 120                 140
Enterobacteriacae sp.CGGGCTCAAC CTGGGAACTG CATTCGAAAC TGGCAGGCTA GAGTCTTGTA GAGGGGGGTA GAATTCCAGG 150
B156                  CGGGCTCAAC CTGGGAACTG CATTCGAAAC TGGCAGGCTA GAGTCTTGTA GAGGGGGGTA GAATTCCAGG 150
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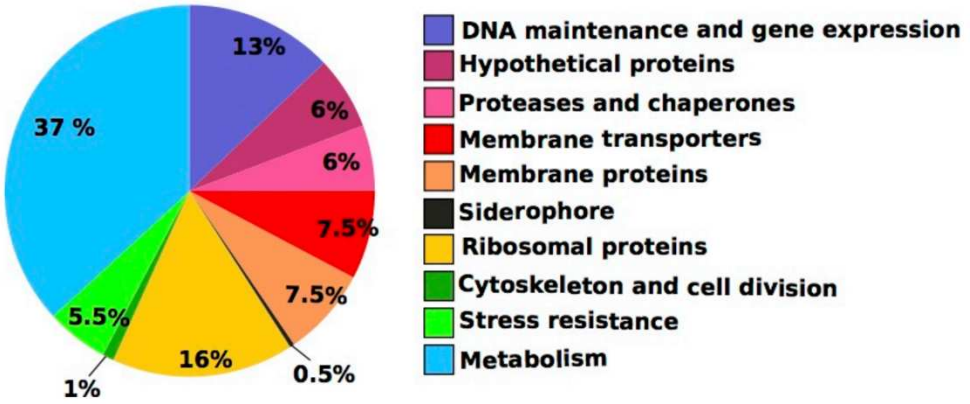
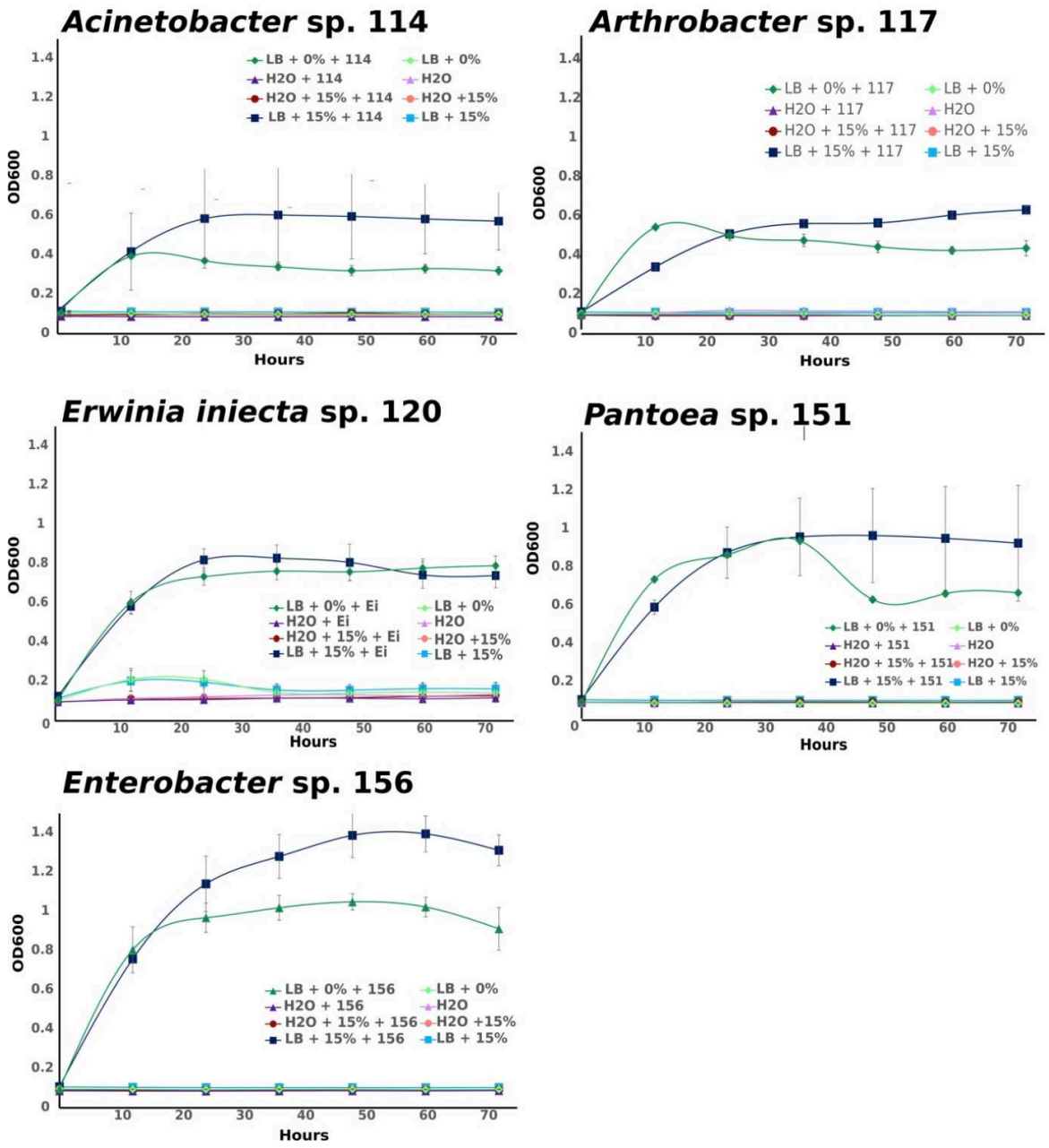
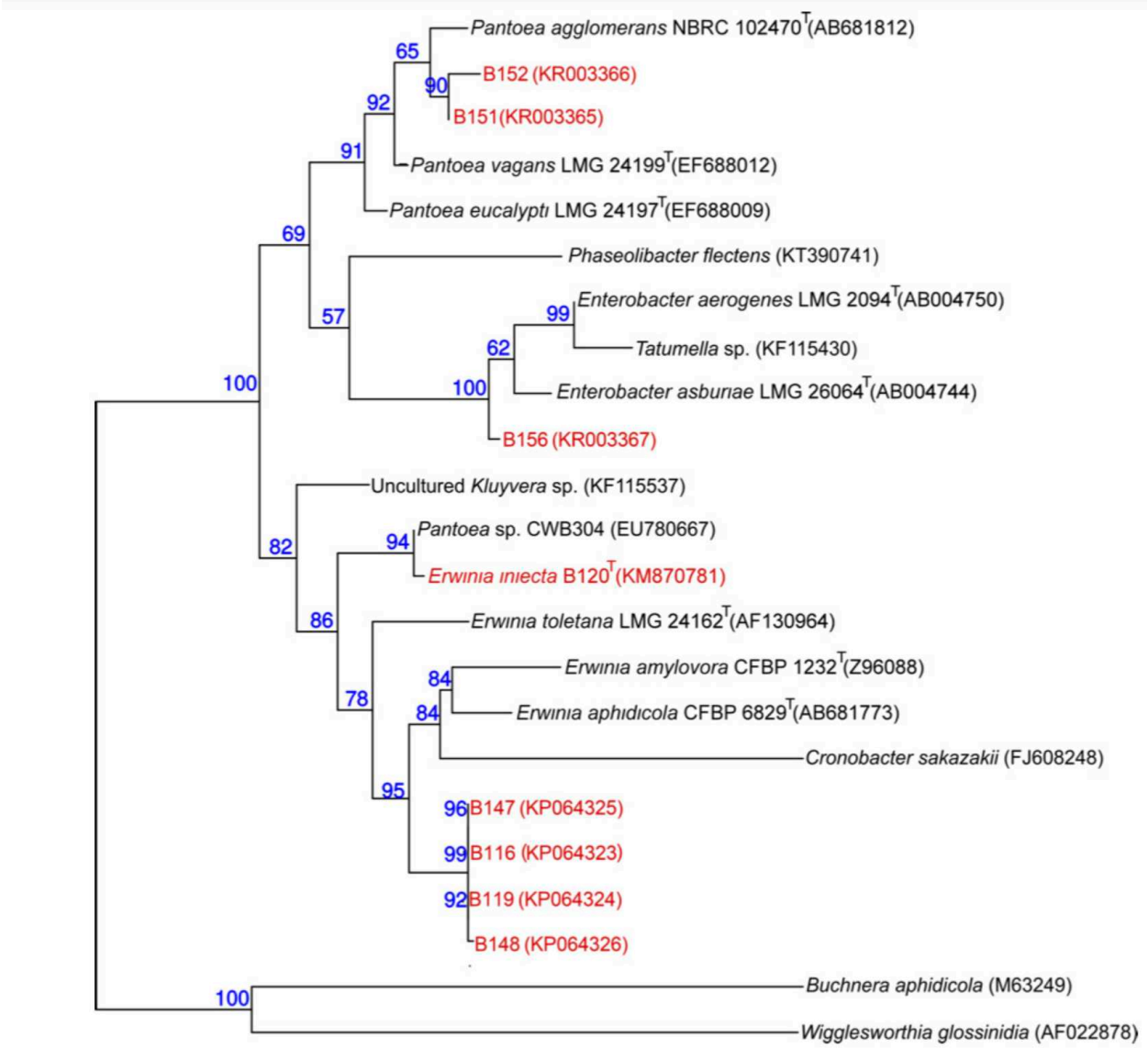
Supplementary Fig. S2.2. Nucleotide alignments of sequences identified in the analysis of the 16S rRNA amplicon sequencing to genomes of bacteria isolated off D. noxia. Sequences were only characterized to the family or genus level when taxonomy was added from the Greengenes database.



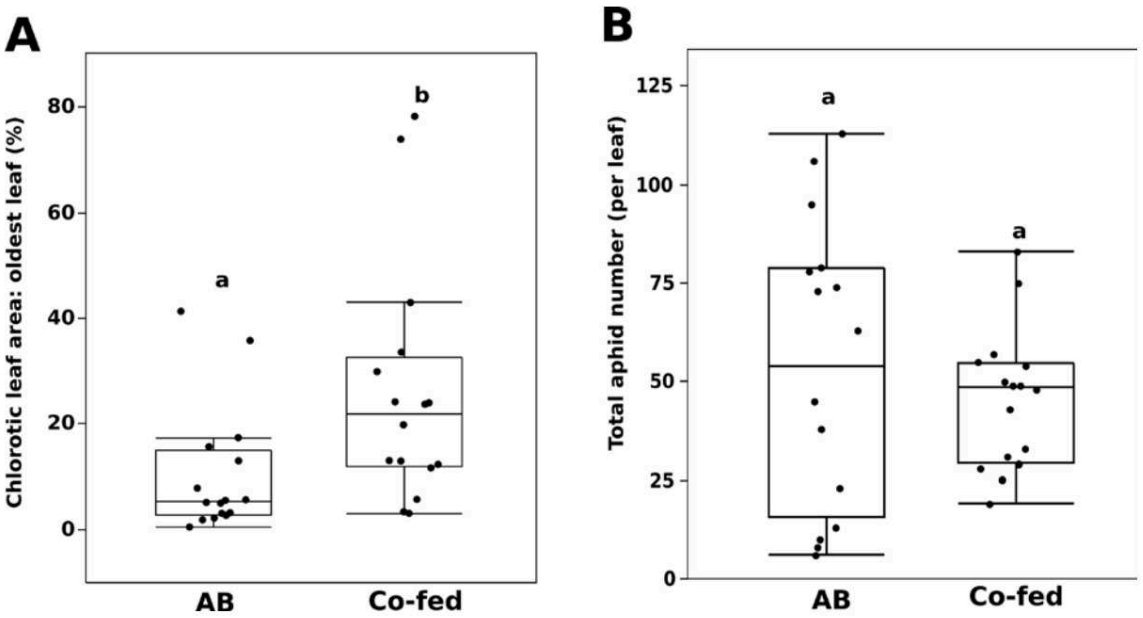
Supplementary Fig. S2.3. *D. noxia* salivary protein functional annotations. Proteins were collected from artificial diets fed on by biotype 1 and 2 for 48 h. Proteins were identified using Mud-PIT analysis and quantified using iTRAQ analysis. Functions of the proteins were predicted by BLAST analysis.



Supplementary Fig. S2.4. *D. noxia*-associated bacteria were cultured in different media with or without sucrose. Media included: Luria broth (LB), LB with 15% sucrose, sterile distilled water, sterile distilled water with 15%.

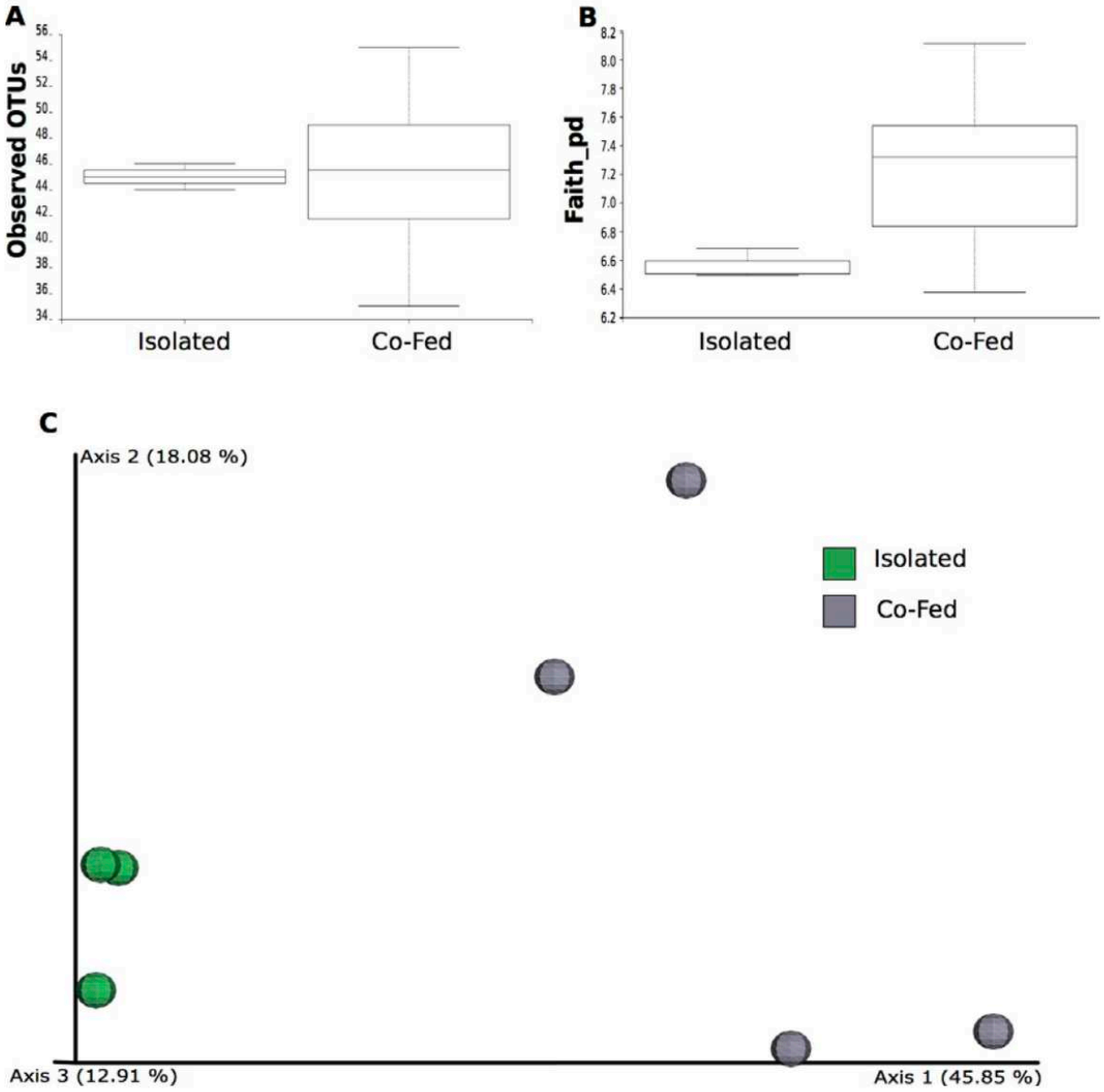


Supplementary Fig. S2.5. Maximum-likelihood trees built from sequences corresponding to the PCR product FD1-RP2 of bacteria isolated from artificial media fed on by *D. noxia* (a), crushed *D. noxia* (b) or wheat leaves after exposure to *D. noxia* (c). Bacterial isolates from this study are shown in red. GenBank accession numbers are given in parentheses. Type strains are indicated by a superscript T.



Supplementary Fig. S2.6 Percent chlorosis of susceptible wheat after infestation of *D. noxia* biotype 2 placed on the second leaf of 14-day old wheat plants. (A) Percent chlorosis of the oldest leaf was determined on 'TAM 107' plants either infested with untreated aphids (control) or aphids treated with antibiotics (AB) (50 ug/ml rifampicin and 50 ug/ml chlortetracycline); (B) Total aphid number after duration of the experiment. The central horizontal line in each box corresponds to the sample median. Minimum and maximum observations (not including outliers) are indicated by the horizontal line at either end of the whiskers, while the end of the box represents the interquartile range (between 25% and 75%). Circles represent individual measurements of four independent experiments; circles are jittered to show all data points.

A t-test was used to compare the number of aphids after two weeks between the antibiotic treatment and the no antibiotic control. We used an ANCOVA to assess the effects of the antibiotic treatment on the logit-transformed proportion of chlorotic leaf tissue that developed after adjusting for the number of aphids per leaf (the covariate).



Supplementary Fig. S2.7. Alpha diversity in 'isolated' and 'co-fed' *D. noxia* microbiome as measured by (A) 16S rRNA gene observed operational taxonomic units (OTUs) and (B) Alpha diversity of *D. noxia* as measured by Faith's Phylogenetic Diversity. 'Isolated' aphids show less phylogenetic diversity than 'co-fed' aphids. (C). Bray-Curtis dissimilarity matrix shows beta diversity between treatments. 'Isolated' and 'co-fed' aphids group separately showing differences in microbial communities.

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A.2 Supplementary Material Chapter 3: *ERWINIA INIECTA* SP. NOV., ISOLATED FROM RUSSIAN WHEAT APHIDS (*DIURAPHIS NOXIA*)

Supplementary Table S3.1. Primers used in this study to amplify housekeeping genes

Gene	Primer forward	Primer reverse	Annealing temperature	Amplicon size	Source
rrs	FD1 ; agagtttgatcctggctcag	RP2 ; acggctaccttgttacgactt	54.3°C	1500 bp	(Weisburg et al., 1991)
atpD	atpD_F; gtctgccaacagtggacctt	atpD_R ; cccagcagggctgatacttc	55.0°C	700 bp	This study
infB	infB_F ; atgccacagaccatcgaagc	infB_R ; gtcacgtaccacggttgct	55.0°C	700 bp	This study
gyrB	gyrB_F ; tcatcagcaggtttacgtacatgg	gyrB_R ; tctcgactgcggtttttacttcaga	53.0°C	2000 bp	This study
rpoB	rpoB_F ; gatcaacgccaagccgatct	rpoB_R ; ctccaggaacggaatcaggg	53.0°C	500 bp	This study

Supplementary Table S3.2. Sequence accession numbers of the strains used for the MultiLocus Sequence Analysis.

Species Strains	GenBank accession numbers 16S rRNA (*rrs*)	*atpD*	*gyrB*	*infB*	*rpoB*
E. amylovora					
CFBP 1232^{T} = LMG 2024^{T}	Z96088	HQ393596	FJ617419	HQ393620	HQ393632
CFBP 1430	FN434113	FN434113	FN434113	FN434113	FN434113
ATCC 49946	FN666575-7	FN666575-7	FN666575-7	FN666575-7	FN666575-7
LMG 2085	-	JF311449	JF311562	JF311675	JF311788
E. aphidicola					
LMG 26028	-	JF311464	JF311577	JF311690	JF311803
LMG 24877^{T}	FN547376	FN547378	FN547377	FN547373	FN547374
E. billingiae					
LMG 2619	-	JF311451	JF311564	JF311677	JF311790
LMG 2613^{T}	JN175337	EU145259	EU145275	EU145291	EU145307
E. mallotivora					
LMG 1270	-	HQ393590	JF311566	JF311679	JF311792
ATCC 29573^{T} = LMG 2708^{T}	AB242909	HQ393589	HQ393601	HQ393613	HQ393625
E. oleae					
LMG 25321	-	HM439616	HM439617	HM439618	HM439619
DAPP-PG 531^{T}	GU810925	GU991653	GU991654	GU991655	GU991656
E. papayae					
CFBP 5300	-	JF311467	JF311580	JF311693	JF311806
CFBP 11606^{T} = NCPPB 4294^{T}	NR_042748	HQ393588	HQ393600	HQ393612	HQ393624
E. persicina					
ICMP 15602	-	JF311460	JF311573	JF311686	JF311799
LMG 11254^{T}	AB681774	HQ393598	HQ393610	HQ393622	HQ393634
E. piriflorinigrans					
CFBP 5888^{T}	GQ405202	JF311469	JF311582	JF311695	JF311808
CFBP 5885	-	JF311471	JF311584	JF311697	JF311810
E. psidii					
LMG 7035	-	HQ393593	HQ393605	HQ393617	HQ393629
LMG 7039^{T}	JQ809696	HQ393594	HQ393606	HQ393618	HQ393630

E. pyrifoliae					
ICMP 13250	-	JF311575	JF311575	JF311688	JF311801
DSM 12163^{I}	AB242880	HQ393597	FJ617420	HQ393621	HQ393633
E. rhapontici					
LMG 2648	-	JF311456	JF311569	JF311682	JF311795
LMG 2688^{I}	AJ233417	EF988751	EF988838	EF988924	EF989010
E. tasmaniensis					
NCPPB 4358	-	HQ393595	HQ393607	HQ393619	HQ393631
ET1/99^{I}	CU468135	NC_010694	NC_010694	NC_010694	NC_010694
E. toletana					
CFBP 6644	-	JF311475	JF311588	JF311701	JF311814
LMG 24162^{I}	FR870447	EU145258	EU145274	EU145290	EU145306
CFBP 6640	AF130962	JF311474	JF311587	JF311700	JF311813
CFBP 6630	AF130909	JF311473	JF311586	JF311699	JF311812
DAPP-PG 735	AOCZ00000000	AOCZ00000000	AOCZ00000000	AOCZ00000000	AOCZ00000000
E. tracheiphila					
LMG 5020	-	JF311457	JF311570	JF311683	JF311796
LMG 2906^{I}	NR_044924	HQ393591	HQ393603	HQ393615	HQ393627
PSU 1	NZ_APJK00000000	NZ_APJK00000000	NZ_APJK00000000	NZ_APJK00000000	NZ_APJK00000000
E. typographi					
DSM 24223	HQ452956	JF508972	-	JF508973	JF508971
DSM 22678^{I}	NR_117446	HQ620539	-	HQ620540	HQ620541
E. uzenensis					
YPPS 951^{I}	NR_113061	-	-	-	-
P. calida					
1400/07^{I}	GQ367478	GQ367477	GQ367480	GQ367476	GQ367479
P. cypripedii					
LMG 2657^{T}	AJ233413	FJ187825	FJ187830	FJ187835	FJ187835
P. dispersa					
LMG 2603^{I}	FJ756349	EF988731	EF988818	EF988904	EF988990
P. eucrina					
LMG 2781^{I}	FJ611863	EU145255	EU145271	EU145287	EU145303
P. gaviniae					

LMG 25382[T]	GQ367483	GQ367482	GQ367485	GQ367481	GQ367484
T. morbirosei					
LMG 23360[T]	EU344769	EU344756	EU344760	EU344764	EU344768
T. punctata					
LMG 22050T	EF688006	EF988716	EF988803	EF988889	EF988975
T. terrea					
LMG 22051T	EF688007	EF988717	EF988804	EF988890	EF988976
E. coli					
K-12 MG1655	NC_000913.3	NC_000913.3	NC_000913.3	NC_000913.3	NC_000913.3
E. iniecta					
B120T	KM870781	-	-	-	-
B137	KM870782	-	-	-	-
B149	KM870783	-	-	-	-
B150	KM870784	-	-	-	-

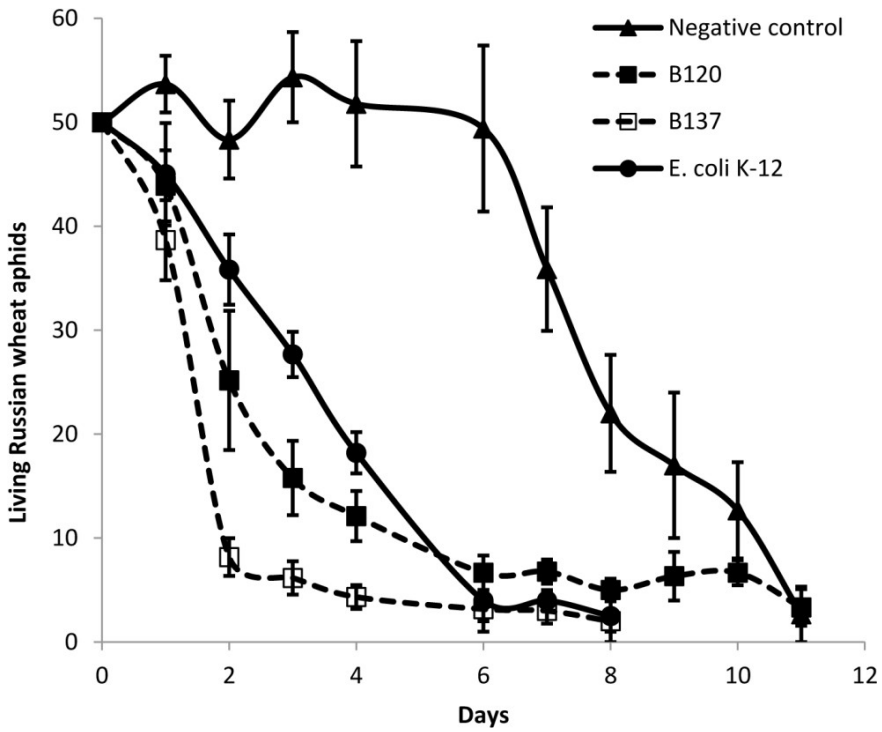
[T] = type strain

Supplementary Table S3.3. Percentages of Average Nucleotide Identity (ANI) between available genome sequences of *Erwinia* type strains.

Query	**Subject** *Erwinia amylovora* CFBP 1232^T	*Erwinia billingiae* LMG 2613^T	*Erwinia piriflorinigrans* CFBP 5888^T	*Erwinia pyrifoliae* CFBP 4172^T	*Erwinia tasmaniensis* Et1/99^T	*Erwinia iniecta* B120^T
Erwinia amylovora CFBP 1232^T		**76.81**	**84.35**	**90.2**	**84.32**	**76.08**
Erwinia billingiae LMG 2613^T	**75.59**		**75.57**	**77.03**	**76.95**	**77.02**
Erwinia piriflorinigrans CFBP 5888^T	**84.44**	**76.8**		**86.25**	**88.98**	**76.18**
Erwinia pyrifoliae CFBP 4172^T	**89.75**	**77.38**	**85.6**		**85.48**	**76.42**
Erwinia tasmaniensis Et1/99^T	**83.79**	**77.34**	**88.45**	**85.59**		**76.17**
Erwinia iniecta B120^T	**75.29**	**77.02**	**75.29**	**76.43**	**76.17**	

Supplementary Table S3.4. Metabolic activities of four *E. iniecta* strains (B120^T, B137, B149 and B150) revealed by Biolog GN microplates. For every test the name of the metabolic activity tested and the percentage of strains showing a positive result is indicated: (-),0-10 % strains positive; (d), 11-89 % strain positive; (+), >90 % strains positive.

Water	Dextrin	D-Maltose	D-Trehalose	D-Cellobiose	Gentiobiose	Sucrose	D-Turanose	Stachyose	Positive Control	pH 6	pH 5
	100 (+)	100 (+)	75 (d)	100 (+)	100 (+)	100 (+)	0 (-)	0 (-)	100 (+)	100 (+)	100 (+)
D-Raffinose	**α-D-Lactose**	**D-Melibiose**	**β-Methyl-DGlucoside**	**D-Salicin**	**N-Acetyl-DGlucosamine**	**N-Acetyl-β-DMannosamine**	**N-Acetyl-DGalactosamine**	**N-Acetyl Neuraminic Acid**	**1% NaCl**	**4% NaCl**	**8% NaCl**
100 (+)	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)	75 (+)	0 (-)	0 (-)	100 (+)	100 (-)	100 (-)
α-D-Glucose	**D-Mannose**	**D-Fructose**	**D-Galactose**	**3-Methyl Glucose**	**D-Fucose**	**L-Fucose**	**L-Rhamnose**	**Inosine**	**1% Sodium Lactate**	**Fusidic Acid**	**D-Serine**
100 (+)	100 (+)	100 (+)	100 (+)	25 (d)	100 (+)	0 (-)	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)
D-Sorbitol	**D-Mannitol**	**D-Arabitol**	**myo-Inositol**	**Glycerol**	**D-Glucose-6-PO4**	**D-Fructose-6-PO4**	**D-Aspartic Acid**	**D-Serine**	**Troleandomycin**	**Rifamycin SV**	**Minocycline**
25 (d)	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)	0 (-)	0 (-)	100 (+)	100 (+)	0 (-)
Gelatin	**Glycyl-L-Proline**	**L-Alanine**	**L-Aspartic Acid**	**L-Arginine**	**L-Glutamic Acid**	**L-Histidine**	**L-Pyroglutamic Acid**	**L-Serine**	**Lincomycin**	**Guanidine HCl**	**Niaproof 4**
0 (-)	100 (+)	100 (+)	0 (-)	100 (+)	100 (+)	75 (d)	25 (d)	100 (+)	100 (+)	100 (+)	100 (+)
Pectin	**D-Galacturonic Acid**	**L-Galactonic Acid Lactone**	**D-Gluconic Acid**	**D-Glucuronic Acid**	**Glucuronamide**	**Mucic Acid**	**Quinic Acid**	**D-Saccharic Acid**	**Vancomycin**	**Tetrazolium Violet**	**Tetrazolium Blue**
100 (+)	100 (+)	50 (d)	100 (+)	100 (+)	100 (+)	0 (-)	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)
p-Hydroxy-Phenylacetic Acid	**Methyl Pyruvate**	**D-Lactic Acid Methyl Ester**	**L-Lactic Acid**	**Citric Acid**	**α-Keto-Glutaric Acid**	**D-Malic Acid**	**L-Malic Acid**	**Bromo-Succinic Acid**	**Nalidixic Acid**	**Lithium Chloride**	**Potassium Tellurite**
0 (-)	100 (+)	0 (-)	100 (+)	0 (-)	50 (d)	0 (-)	100 (+)	100 (+)	100 (+)	100 (+)	100 (+)
Tween 40	**γ-Amino-Butyric Acid**	**α-Hydroxy-Butyric Acid**	**β-Hydroxy-D,LButyric Acid**	**α-Keto-Butyric Acid**	**Acetoacetic Acid**	**Propionic Acid**	**Acetic Acid**	**Formic Acid**	**Aztreonam**	**Sodium Butyrate**	**Sodium Bromate**
0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	100 (+)	0 (-)	100 (+)	0 (-)	0 (-)



Supplementary Fig. S3.1. Survival of Russian wheat aphid following ingestion of *Erwinia iniecta* strains B120^{T}, B137 or *E. coli* K-12.

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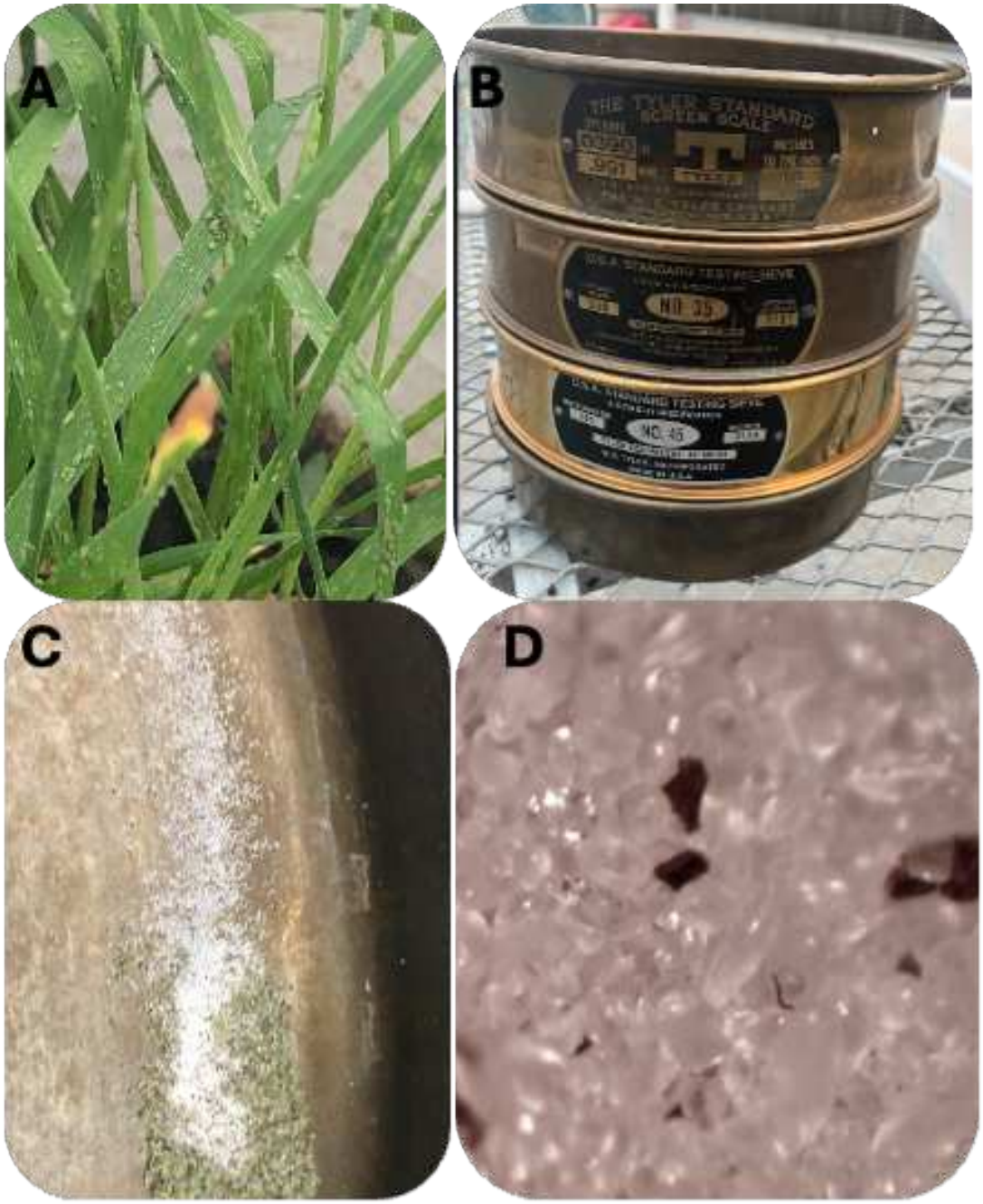
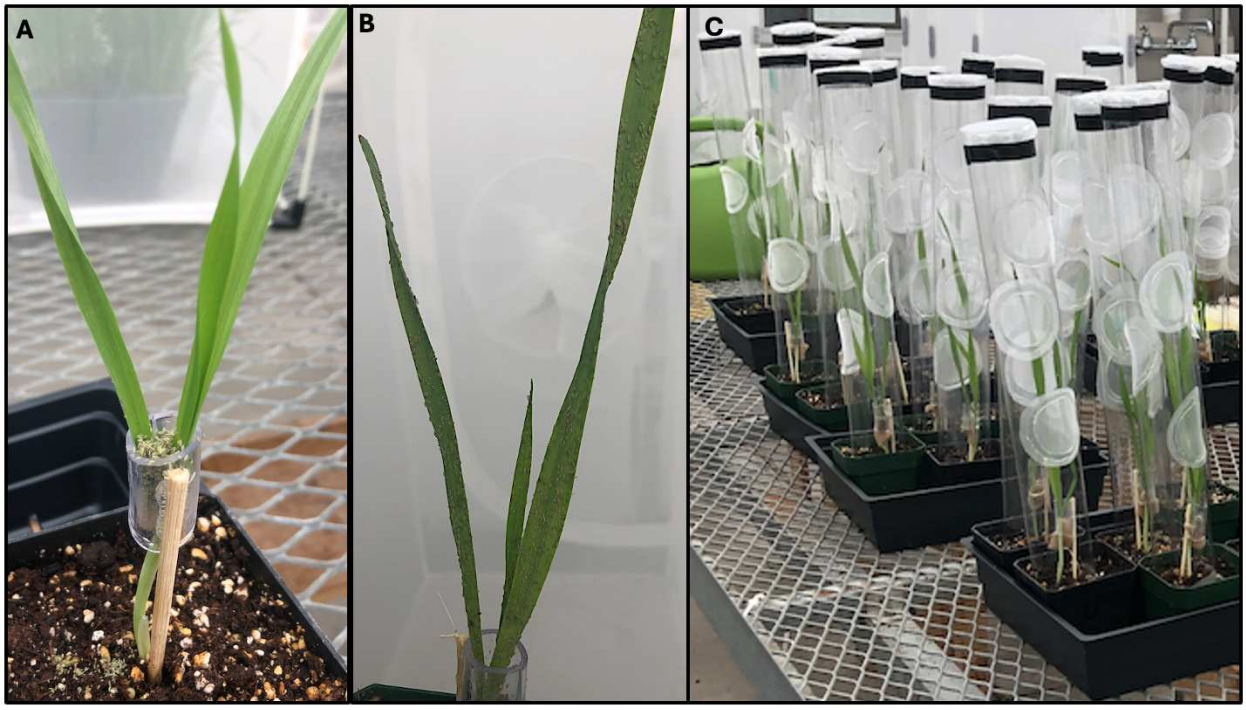
A.3 Supplementary Materials Chapter 4: BACTERIA ASSOCIATED WITH RUSSIAN WHEAT APHID HONEYDEW MODULATE DEFENSE RESPONSE GENE PATHWAYS IN BARLEY

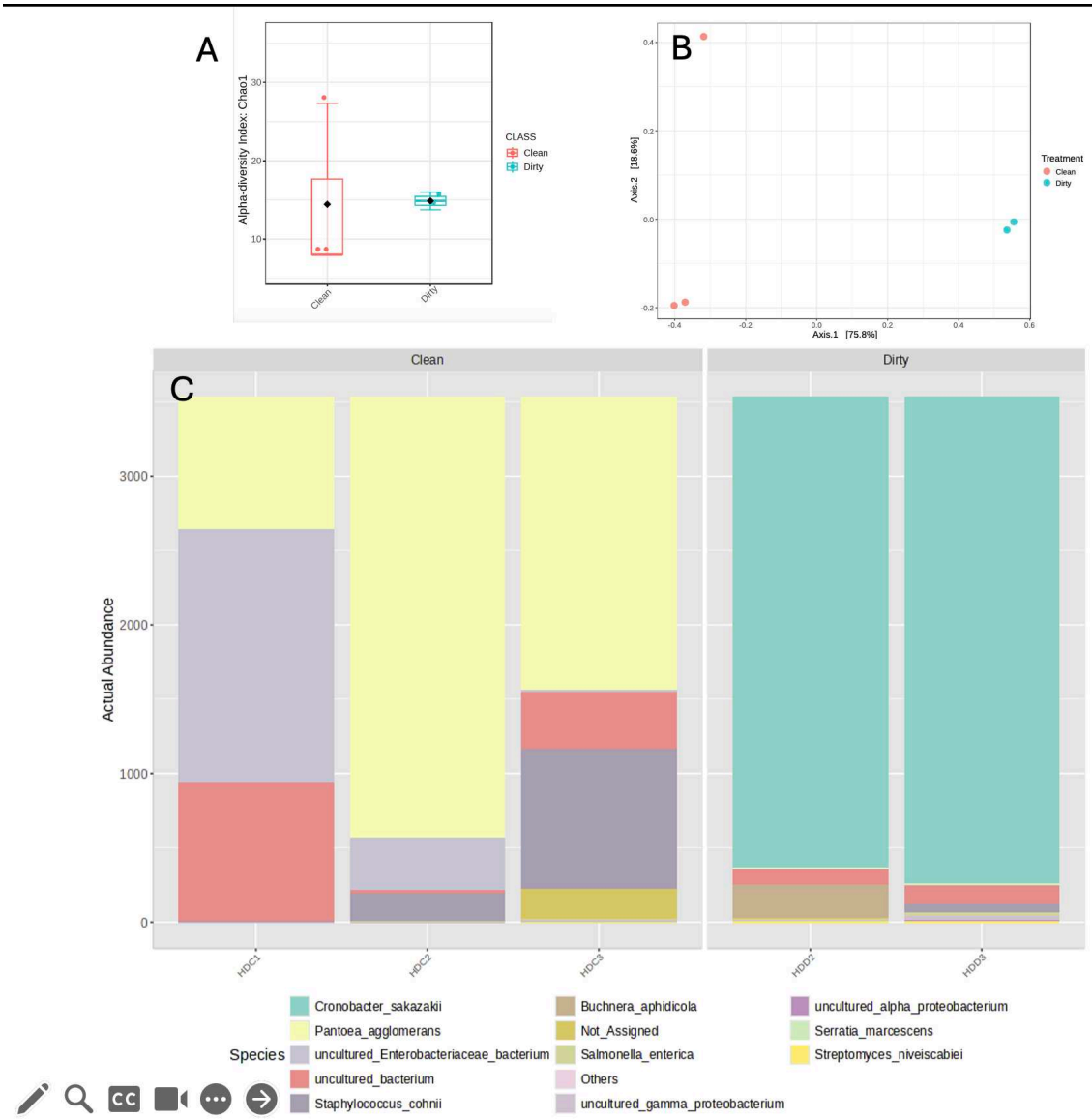


Supplementary Fig. S4.1: Collection of RWA honeydew. Leaves from heavily aphid-infested barley Wheat plants (A) were collected, and agitated over a series of sieves (No. 16, 35 and 45 respectively) to filter out large aphids, debris and soil. Honeydew was then separated from

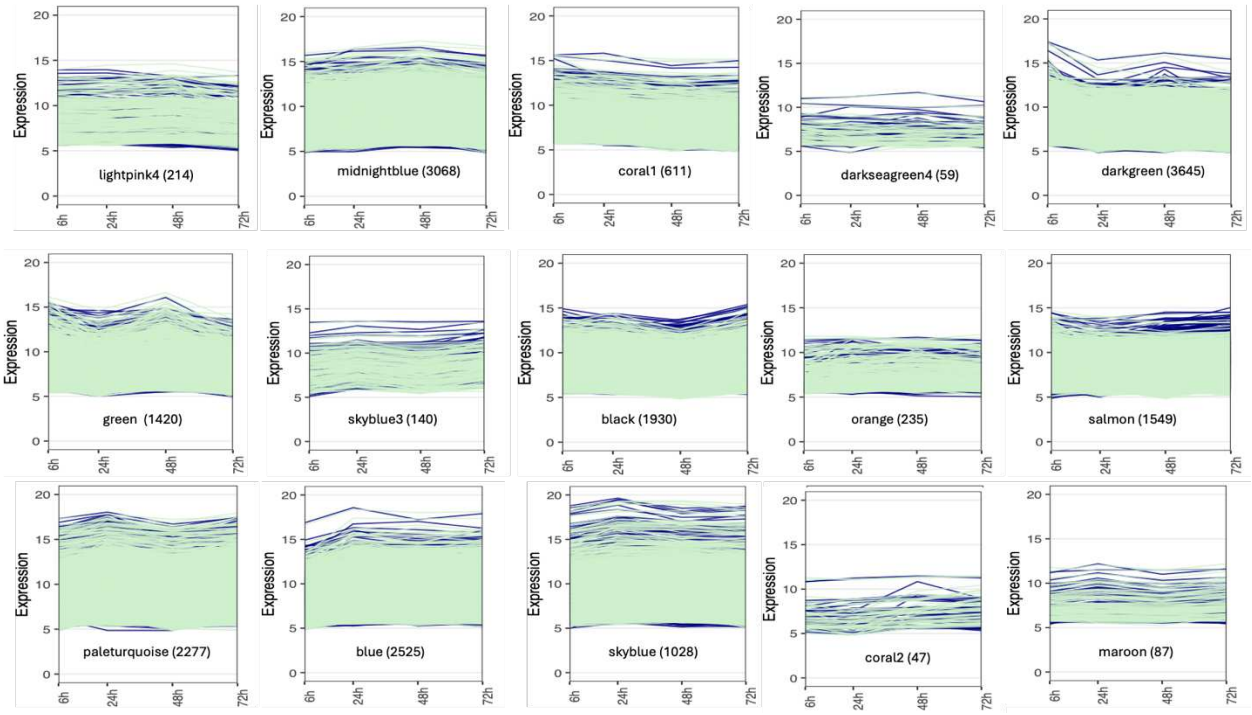
small aphids (C). Although some debris persisted in the final honeydew product (D), samples were free of aphids.



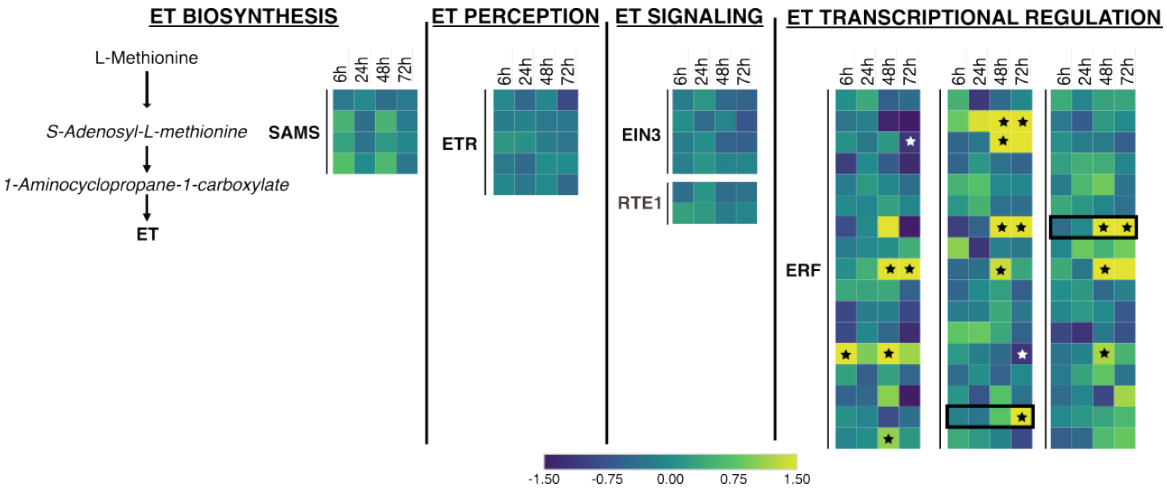
Supplementary Fig. S4.2: Experimental setup for Russian wheat aphid transcriptomic experiment (A) Aphids were introduced into 1 x 1 inch piece of vinyl tubing positioned around the base of each plant (B) Thirty minutes post-infestation, aphids disperse across leaf surface. (C) Vented plastic sleeves were placed over each plant to prevent unintended herbivore access while maintaining airflow.



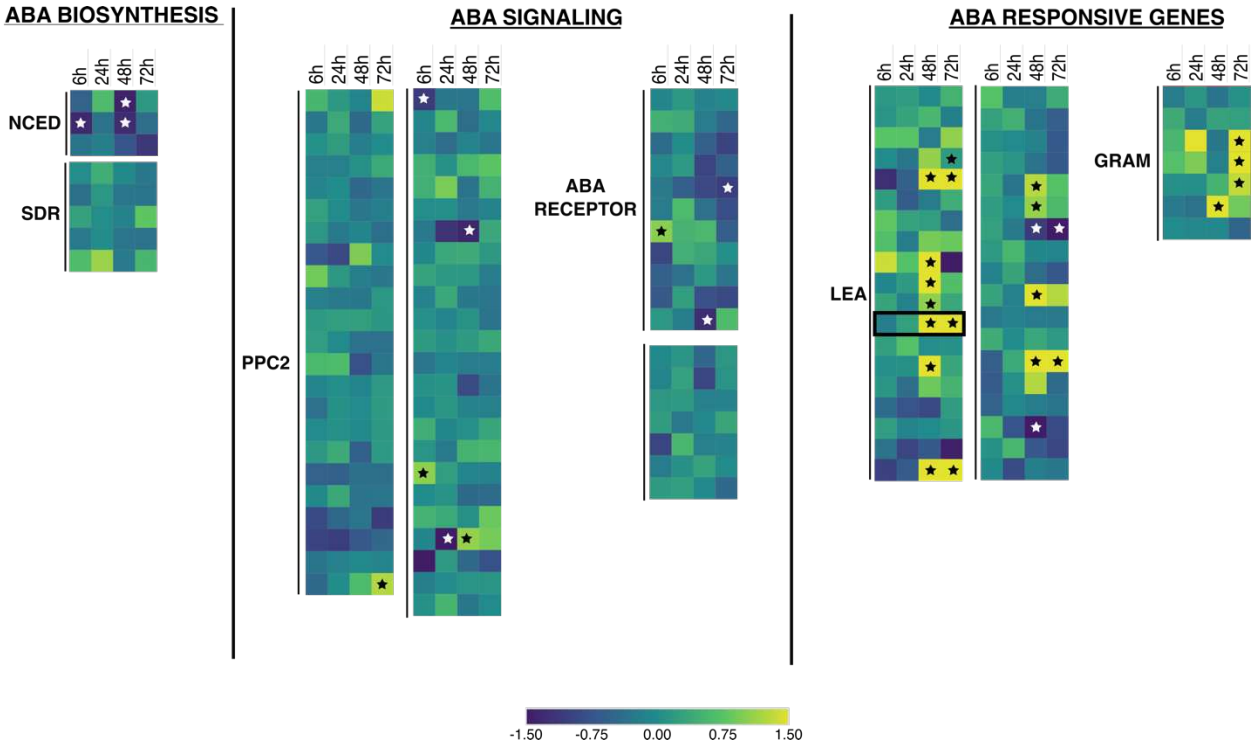
Supplementary Fig. S4.3: Species richness **(A)** and beta diversity **(B)** of RWA honeydew collected from isolated and co-fed aphids. **(C)** Genus level taxonomy of RWA honeydew.



Supplementary Fig. S4.4. Gene expression profiles for each module identified by WGCNA. The plots show module-specific gene expression levels over time (6, 24, 48, and 72 h) in response to co-fed (dark blue lines) or isolated (mint green lines) honeydew treatment. Each panel represents a module, with the module name and the number of genes in parentheses.



Supplementary Fig. S4.5. Expression patterns in the ethylene (ET) biosynthesis and signaling pathways in barley plants infiltrated with honeydew treatments collected from co-fed and isolated aphids. Colors indicated log2 fold changes (co-fed compared to isolated), ranging from purple (low expression) to yellow (high expression). Black stars denote upregulated DEGs, white stars indicate downregulated DEGs, and boxed genes represent hub genes in the WGCNA Salmon module.



Supplementary Fig. S4.6. Expression patterns in the abscisic acid (ABA) biosynthesis and signaling pathways in barley plants infiltrated with honeydew treatments collected from co-fed and isolated aphids. Colors indicated log2 fold changes (co-fed compared to isolated), ranging from purple (low expression) to yellow (high expression). Black stars denote upregulated DEGs, white stars indicate downregulated DEGs, and boxed genes represent hub genes in the WGCNA Salmon module.

Supplementary Table S4.1. Top 15% Hub Genes in Salmon Module

GeneID	geneDescription	GS.HD	p.GS.HD	MM.salmon	p.MM.salmon
HORVU.MOREX.r3.2HG0097740	ARM repeat superfamily protein	0.52813998	0.01151888	0.87920851	7.18E-08
HORVU.MOREX.r3.1HG0093110	Receptor-like protein kinase	0.48168558	0.02321363	0.85414373	4.25E-07
HORVU.MOREX.r3.3HG0264790	Abscisic acid-responsive protein	0.4221223	0.05035525	0.88204566	5.73E-08
HORVU.MOREX.r3.5HG0510250	Dirigent protein	0.6612359	0.00080585	0.80898051	5.17E-06
HORVU.MOREX.r3.3HG0322580	Glycosyltransferase	0.60754704	0.00270865	0.8021003	7.14E-06
HORVU.MOREX.r3.2HG0102660	Serine/threonine-protein kinase	0.60751115	0.00271065	0.89790106	1.45E-08
HORVU.MOREX.r3.7HG0657430	Hexosyltransferase	0.58345814	0.0043661	0.85297378	4.58E-07
HORVU.MOREX.r3.7HG0702290	Tryptophan synthase beta chain	0.5670741	0.0059206	0.90233812	9.47E-09
HORVU.MOREX.r3.6HG0630920	NBS-LRR disease resistance protein-like	0.55718967	0.00706302	0.93488715	1.89E-10
HORVU.MOREX.r3.2HG0203400	Vacuolar cation/proton exchanger, putative	0.55680857	0.00711047	0.87236793	1.21E-07

HORVU.MOREX.r3.4HG0334480	Nodulin-like / Major Facilitator Superfamily protein	0.55262625	0.00764884	0.84796593	6.26E-07
HORVU.MOREX.r3.2HG0191960	Transporter-related family protein	0.54963011	0.00805486	0.88719001	3.75E-08
HORVU.MOREX.r3.4HG0406450	Purine permease-like protein	0.54494225	0.00872569	0.85075514	5.27E-07
HORVU.MOREX.r3.3HG0283580	Cellulose synthase	0.5440589	0.00885711	0.88950117	3.08E-08
HORVU.MOREX.r3.3HG0237390	3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase	0.54334994	0.00896376	0.94639026	2.83E-11
HORVU.MOREX.r3.3HG0281260	Receptor-like kinase	0.53966506	0.00953522	0.91948492	1.48E-09
HORVU.MOREX.r3.3HG0242050	Alpha/beta-Hydrolases superfamily protein	0.53684634	0.00999228	0.96249023	8.52E-13
HORVU.MOREX.r3.5HG0523050	Phosphate transporter	0.53526454	0.01025654	0.85606355	3.75E-07
HORVU.MOREX.r3.3HG0240640	Glycosyltransferase	0.53459151	0.01037071	0.91091037	3.92E-09
HORVU.MOREX.r3.7HG0709580	ADP-ribosylation factor family protein	0.53376625	0.01051212	0.94107553	7.13E-11
HORVU.MOREX.r3.7HG0743270	WRKY transcription factor	0.52962667	0.0112455	0.80375072	6.61E-06
HORVU.MOREX.r3.3HG0266950	Calmodulin-binding protein	0.52602905	0.01191638	0.84173022	9.10E-07

HORVU.MOREX.r3.4HG0383890	DUF538 family protein (Protein of unknown function, DUF538)	0.5256544	0.01198809	0.94419234	4.20E-11
HORVU.MOREX.r3.3HG0253410	Pectinesterase	0.52546236	0.01202498	0.85384223	4.33E-07
HORVU.MOREX.r3.5HG0520830	biological regulation (GO:0065007)	0.52325453	0.01245581	0.90847409	5.08E-09
HORVU.MOREX.r3.2HG0213820	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family, putative	0.5187993	0.01336358	0.92373609	8.74E-10
HORVU.MOREX.r3.3HG0283700	Basic helix-loop-helix (BHLH) family transcription factor	0.51817704	0.01349455	0.8599781	2.90E-07
HORVU.MOREX.r3.6HG0574250	60 kDa chaperonin	0.51471959	0.01424141	0.82927653	1.84E-06
HORVU.MOREX.r3.7HG0746590	magnesium transporter NIPA (DUF803)	0.51435523	0.01432204	0.85674918	3.59E-07
HORVU.MOREX.r3.7HG0648480	Lectin receptor kinase	0.51245056	0.01474958	0.89986727	1.20E-08
HORVU.MOREX.r3.2HG0160240	Exocyst complex component exo70a1	0.5101951	0.0152692	0.95346544	7.09E-12

HORVU.MOREX.r3.3HG0285900	Ribose-phosphate pyrophosphokinase, putative	0.50919647	0.01550396	0.82502868	2.31E-06
HORVU.MOREX.r3.1HG0071970	RING zinc finger protein	0.5082545	0.01572808	0.87845672	7.62E-08
HORVU.MOREX.r3.1HG0083340	AP2/B3 transcription factor family protein	0.50746283	0.01591846	0.85682708	3.57E-07
HORVU.MOREX.r3.7HG0703490	Amino acid transporter, putative	0.50430668	0.01669612	0.85238631	4.75E-07
HORVU.MOREX.r3.5HG0538180	Pleiotropic drug resistance ABC transporter	0.5037905	0.01682618	0.86074733	2.75E-07
HORVU.MOREX.r3.7HG0667900	Metalloendoproteinase 1	0.50326766	0.01695875	0.9554977	4.57E-12
HORVU.MOREX.r3.2HG0208580	Receptor-kinase, putative	0.50302581	0.01702036	0.86903181	1.54E-07
HORVU.MOREX.r3.1HG0057580	PRA1 family protein	0.50127233	0.01747247	0.94383136	4.47E-11
HORVU.MOREX.r3.3HG0311790	Kinase family protein	0.5010461	0.0175315	0.91758001	1.85E-09
HORVU.MOREX.r3.4HG0389790	Receptor-like kinase	0.4973004	0.01853252	0.94231654	5.80E-11
HORVU.MOREX.r3.5HG0433130	Calmodulin	0.49655332	0.01873758	0.83166329	1.61E-06
HORVU.MOREX.r3.2HG0182020	Phenylalanine ammonia-lyase	0.49621614	0.01883073	0.80585852	5.99E-06
HORVU.MOREX.r3.2HG0131780	Lectin receptor kinase	0.49602424	0.0188839	0.82724967	2.05E-06

HORVU.MOREX.r3.5HG0460570	Glycosyltransferase	0.49533786	0.01907511	0.94802844	2.09E-11
HORVU.MOREX.r3.5HG0430760	Kinase, putative	0.4951319	0.01913278	0.81431068	3.99E-06
HORVU.MOREX.r3.2HG0193650	Ubiquinol oxidase	0.49437939	0.01934471	0.89503621	1.89E-08
HORVU.MOREX.r3.4HG0406630	Classical arabinogalactan protein 5	0.49398939	0.01945529	0.90962792	4.50E-09
HORVU.MOREX.r3.3HG0303900	Trichome birefringence-like protein	0.49295202	0.01975189	0.94670536	2.68E-11
HORVU.MOREX.r3.2HG0138340	Xylanase inhibitor protein 1	0.49260363	0.01985231	0.84300674	8.44E-07
HORVU.MOREX.r3.5HG0522100	Germin-like protein	0.49150314	0.02017223	0.95983088	1.67E-12
HORVU.MOREX.r3.5HG0520940	ATP-dependent zinc metalloprotease FtsH	0.49064848	0.02042353	0.89087433	2.74E-08
HORVU.MOREX.r3.3HG0245610	Ring finger protein, putative	0.48878976	0.02097876	0.88118583	6.14E-08
HORVU.MOREX.r3.7HG0746660	Eukaryotic aspartyl protease family protein	0.48831307	0.02112309	0.84066289	9.69E-07
HORVU.MOREX.r3.5HG0484690	Calcium-binding family protein	0.48791425	0.02124446	0.87833918	7.69E-08
HORVU.MOREX.r3.6HG0545990	Cysteine-rich receptor kinase	0.48757286	0.02134879	0.83398781	1.42E-06
HORVU.MOREX.r3.5HG0430620	Protein kinase	0.48638794	0.02171411	0.92968197	3.98E-10

HORVU.MOREX.r3.5HG0518060	Glycosyltransferase	0.48606111	0.02181575	0.89034962	2.86E-08
HORVU.MOREX.r3.3HG0274050	DWNN domain, a CCHC-type zinc finger	0.48449411	0.02230839	0.89477467	1.93E-08
HORVU.MOREX.r3.7HG0704190	myelin-associated oligodendrocyte basic protein	0.48224622	0.02303062	0.87633758	8.97E-08
HORVU.MOREX.r3.1HG0071920	Receptor protein kinase, putative	0.48201002	0.02310758	0.90980982	4.41E-09
HORVU.MOREX.r3.4HG0333220	WRKY transcription factor	0.48184148	0.02316262	0.80384287	6.59E-06
HORVU.MOREX.r3.7HG0648470	Lectin receptor kinase	0.48181125	0.02317251	0.83383339	1.43E-06
HORVU.MOREX.r3.4HG0336870	Microsomal glutathione S-transferase 3	0.47891184	0.02413628	0.98003847	1.67E-15
HORVU.MOREX.r3.7HG0653740	Long-Chain Acyl-CoA Synthetase	0.47721597	0.02471466	0.9693087	1.18E-13
HORVU.MOREX.r3.3HG0298710	DUF668 family protein	0.47714907	0.02473771	0.87539506	9.64E-08
HORVU.MOREX.r3.7HG0661490	Stromal cell-derived factor 2	0.47611999	0.02509431	0.92915007	4.28E-10
HORVU.MOREX.r3.5HG0470090	Indole-3-glycerol phosphate synthase	0.47444233	0.02568444	0.87639087	8.94E-08
HORVU.MOREX.r3.5HG0482390	Tetraspanin family protein	0.47353477	0.02600827	0.90355541	8.40E-09

HORVU.MOREX.r3.5HG0503550	Serine/threonine-protein kinase	0.47334742	0.02607552	0.86430318	2.16E-07
HORVU.MOREX.r3.4HG0344970	receptor kinase 1	0.4725697	0.02635617	0.87759002	8.15E-08
HORVU.MOREX.r3.4HG0401170	Blue copper protein	0.47252503	0.02637236	0.81589091	3.69E-06
HORVU.MOREX.r3.2HG0183890	Calcium-dependent lipid-binding domain-containing protein	0.4715807	0.02671654	0.9266859	5.97E-10
HORVU.MOREX.r3.5HG0517040	Syntaxin, putative	0.47068532	0.02704616	0.91750389	1.87E-09
HORVU.MOREX.r3.4HG0379260	Calcium binding protein	0.47030143	0.02718848	0.90141596	1.04E-08
HORVU.MOREX.r3.5HG0475950	cotton fiber-like protein (DUF761)	0.46987844	0.02734597	0.82114734	2.82E-06
HORVU.MOREX.r3.1HG0086260	NAD(P)-binding Rossmann-fold superfamily protein	0.46985179	0.02735592	0.88991901	2.97E-08
HORVU.MOREX.r3.5HG0527720	Receptor protein kinase, putative,expressed	0.46849356	0.02786671	0.89898958	1.31E-08
HORVU.MOREX.r3.5HG0478950	Centromere protein V	0.46750311	0.02824394	0.91871298	1.62E-09
HORVU.MOREX.r3.4HG0412120	Zinc finger (C3HC4-type RING finger) family protein	0.46709187	0.02840176	0.92658351	6.05E-10
HORVU.MOREX.r3.7HG0660720	Ring finger protein, putative	0.4658548	0.02888072	0.88904379	3.21E-08

HORVU.MOREX.r3.6HG0543180	Germin-like protein	0.46440319	0.02945089	0.80712776	5.64E-06
HORVU.MOREX.r3.6HG0617730	Galactoside 2-alpha-L-fucosyltransferase	0.46377633	0.02969985	0.91694902	1.99E-09
HORVU.MOREX.r3.7HG0738010	VQ motif family protein	0.46351573	0.02980384	0.85229393	4.78E-07
HORVU.MOREX.r3.4HG0389730	Anthranilate synthase	0.46254864	0.03019227	0.9512597	1.12E-11
HORVU.MOREX.r3.2HG0183740	Sugar transporter, putative	0.45987824	0.0312857	0.92930262	4.20E-10
HORVU.MOREX.r3.7HG0703460	Amino acid transporter, putative	0.45820637	0.03198605	0.84576546	7.16E-07
HORVU.MOREX.r3.5HG0485940	Hexosyltransferase	0.45807079	0.03204338	0.91252875	3.29E-09
HORVU.MOREX.r3.3HG0302620	Calcium-dependent lipid-binding domain-containing protein	0.4578247	0.03214766	0.80423993	6.46E-06
HORVU.MOREX.r3.5HG0471680	Ferrochelatase	0.45699424	0.03250151	0.93955234	9.15E-11
HORVU.MOREX.r3.5HG0490260	FAD-binding Berberine family protein	0.45693775	0.0325257	0.90266045	9.17E-09
HORVU.MOREX.r3.5HG0512730	Zinc-finger protein	0.45656942	0.03268372	0.8348271	1.35E-06
HORVU.MOREX.r3.3HG0281780	Protein transport protein Sec61 subunit alpha	0.45641069	0.032752	0.92084458	1.25E-09

HORVU.MOREX.r3.6HG0543150	Protein kinase family protein	0.45416173	0.03373165	0.84720106	6.56E-07
HORVU.MOREX.r3.4HG0408170	Protein kinase	0.45383335	0.0338766	0.98161391	7.39E-16
HORVU.MOREX.r3.2HG0200920	Agmatine coumaroyltransferase-2	0.45257444	0.03443686	0.82367687	2.48E-06
HORVU.MOREX.r3.4HG0406980	Ras-related protein	0.45250477	0.03446808	0.92826473	4.83E-10
HORVU.MOREX.r3.2HG0095510	Reductase 1	0.45248603	0.03447648	0.91066564	4.02E-09
HORVU.MOREX.r3.7HG0745000	Amino acid transporter-like protein	0.45157004	0.03488908	0.85121626	5.12E-07
HORVU.MOREX.r3.4HG0413510	UDP-galactose transporter	0.45086015	0.0352115	0.95607276	4.03E-12
HORVU.MOREX.r3.4HG0343710	Calcineurin-like metallo-phosphoesterase superfamily protein	0.44896356	0.03608441	0.95883012	2.13E-12
HORVU.MOREX.r3.6HG0551960	protein kinase family protein	0.44886115	0.03613203	0.85200549	4.87E-07
HORVU.MOREX.r3.2HG0127020	methyl esterase 11	0.44797137	0.0365478	0.96742707	2.12E-13
HORVU.MOREX.r3.4HG0339930	Endoplasmic reticulum oxidoreductin-1	0.446204	0.03738479	0.94944355	1.60E-11
HORVU.MOREX.r3.4HG0393050	U-box domain-containing protein	0.44528715	0.03782487	0.89626209	1.69E-08

HORVU.MOREX.r3.1HG0029360	WRKY transcription factor	0.44455677	0.03817835	0.92306745	9.52E-10
HORVU.MOREX.r3.3HG0287850	Receptor-like protein kinase	0.44430893	0.03829889	0.87659699	8.80E-08
HORVU.MOREX.r3.5HG0474540	Protein transport protein Sec61 subunit alpha	0.44395202	0.03847299	0.91225848	3.38E-09
HORVU.MOREX.r3.2HG0202880	PRLI-interacting factor	0.44390479	0.03849607	0.95593618	4.15E-12
HORVU.MOREX.r3.7HG0682090	Oxysterol-binding protein-like	0.44377863	0.03855779	0.85834925	3.23E-07
HORVU.MOREX.r3.6HG0574410	Receptor-like protein kinase	0.44312998	0.03887634	0.84356699	8.17E-07
HORVU.MOREX.r3.2HG0191770	Ethylene-responsive transcription factor, putative	0.44259041	0.03914289	0.87206842	1.24E-07
HORVU.MOREX.r3.4HG0383900	DUF1262 family protein	0.44229265	0.03929059	0.81516908	3.82E-06
HORVU.MOREX.r3.2HG0200560	WRKY transcription factor	0.44171268	0.03957954	0.82663927	2.12E-06
HORVU.MOREX.r3.5HG0512540	Prolyl 4-hydroxylase alpha subunit, putative	0.44168767	0.03959203	0.91562445	2.32E-09
HORVU.MOREX.r3.2HG0118230	Chaperone dnaJ-like protein	0.4416835	0.03959412	0.88463247	4.65E-08
HORVU.MOREX.r3.3HG0331030	Chaperone protein dnaJ, putative	0.44092741	0.03997341	0.95440062	5.81E-12

HORVU.MOREX.r3.7HG0732170	Calcium-binding family protein	0.4402824	0.04029922	0.91292873	3.14E-09
HORVU.MOREX.r3.6HG0570940	LEM3 (Ligand-effect modulator 3)-like	0.44003085	0.04042685	0.91847748	1.67E-09
HORVU.MOREX.r3.5HG0509810	Calmodulin	0.43962655	0.04063264	0.8659503	1.92E-07
HORVU.MOREX.r3.1HG0009460	polyadenylate-binding protein 1-B-binding protein	0.43907417	0.04091512	0.95997025	1.62E-12
HORVU.MOREX.r3.1HG0064890	ATP-dependent zinc metalloprotease FtsH	0.43885762	0.04102628	0.92111461	1.21E-09
HORVU.MOREX.r3.7HG0723770	BZIP transcription factor protein	0.43884155	0.04103454	0.95490457	5.21E-12
HORVU.MOREX.r3.3HG0270240	ATP-dependent zinc metalloprotease FtsH	0.43879524	0.04105834	0.92170992	1.13E-09
HORVU.MOREX.r3.4HG0383400	Protein COBRA, putative	0.43799642	0.04147068	0.89597921	1.73E-08
HORVU.MOREX.r3.2HG0125430	Jasmonate-zim-domain protein	0.43738517	0.04178838	0.91999137	1.39E-09
HORVU.MOREX.r3.3HG0273390	ATP-dependent zinc metalloprotease FtsH 1	0.43678048	0.04210454	0.86094789	2.71E-07
HORVU.MOREX.r3.4HG0380790	VQ motif-containing protein 1	0.43643886	0.04228397	0.89581171	1.76E-08

HORVU.MOREX.r3.1HG0088330	Ras-related protein, expressed	0.43640507	0.04230175	0.80445221	6.40E-06
HORVU.MOREX.r3.2HG0202280	Amino acid permease	0.4362005	0.04240952	0.87233745	1.21E-07
HORVU.MOREX.r3.2HG0212570	Calreticulin	0.43534952	0.04286013	0.93068527	3.46E-10
HORVU.MOREX.r3.5HG0476020	Ascorbate oxidase	0.43479431	0.04315612	0.87948351	7.03E-08
HORVU.MOREX.r3.4HG0398060	Phosphatase 2C family protein	0.43436529	0.04338593	0.95282718	8.10E-12
HORVU.MOREX.r3.1HG0055890	Proline dehydrogenase	0.43358536	0.04380616	0.84328971	8.30E-07
HORVU.MOREX.r3.1HG0089010	Kinase family protein	0.43323299	0.04399705	0.94889466	1.77E-11
HORVU.MOREX.r3.6HG0571070	VQ motif family protein	0.43261932	0.04433104	0.83989061	1.01E-06
HORVU.MOREX.r3.6HG0627340	Receptor-kinase, putative	0.4322428	0.04453693	0.82657977	2.12E-06
HORVU.MOREX.r3.3HG0242790	Cell number regulator 8	0.43152273	0.04493277	0.92396862	8.49E-10
HORVU.MOREX.r3.2HG0131170	Polyol transporter	0.43136435	0.0450202	0.81990383	3.01E-06
HORVU.MOREX.r3.7HG0743280	WRKY transcription factor	0.42867602	0.0465245	0.91649237	2.10E-09
HORVU.MOREX.r3.5HG0518810	Alpha/beta-Hydrolases superfamily protein, putative	0.42806601	0.0468712	0.85087288	5.23E-07
HORVU.MOREX.r3.1HG0019970	Agmatine coumaroyltransferase-1	0.42787127	0.0469823	0.89135422	2.62E-08

HORVU.MOREX.r3.2HG0107940	MLO-like protein	0.42756416	0.04715794	0.90518655	7.13E-09
HORVU.MOREX.r3.2HG0215660	Kinase, putative	0.42735468	0.04727803	0.92123474	1.19E-09
HORVU.MOREX.r3.7HG0737860	Receptor-kinase, putative	0.42722844	0.04735051	0.96232761	8.89E-13
HORVU.MOREX.r3.1HG0021970	1-aminocyclopropane-1-carboxylate oxidase	0.42591302	0.04811093	0.86175205	2.57E-07
HORVU.MOREX.r3.6HG0609180	Thioredoxin reductase	0.42392712	0.04927682	0.83900949	1.07E-06
HORVU.MOREX.r3.7HG0746410	Glutathione S-transferase	0.42322555	0.04969388	0.89682198	1.60E-08
HORVU.MOREX.r3.4HG0406380	GEM-like protein 1	0.42228505	0.05025726	0.91470793	2.58E-09
HORVU.MOREX.r3.5HG0513630	Lectin receptor kinase	0.42145366	0.05075938	0.80547746	6.10E-06
HORVU.MOREX.r3.2HG0204970	Leucine-rich repeat receptor-like protein kinase family protein	0.42138363	0.05080185	0.82272749	2.60E-06
HORVU.MOREX.r3.2HG0191440	Vacuolar sorting receptor family protein	0.42110348	0.05097203	0.90052924	1.13E-08
HORVU.MOREX.r3.2HG0178020	Chitinase	0.41952817	0.05193715	0.9161722	2.18E-09
HORVU.MOREX.r3.6HG0564580	Lectin receptor kinase	0.41866034	0.05247482	0.8812132	6.13E-08
HORVU.MOREX.r3.4HG0406580	Classical arabinogalactan protein 5	0.41812273	0.05281005	0.92207693	1.08E-09

HORVU.MOREX.r3.6HG0633710	LysM receptor-like kinase protein	0.41740966	0.05325723	0.95869246	2.20E-12
HORVU.MOREX.r3.4HG0390960	Ankyrin repeat family protein	0.41720584	0.05338558	0.88026198	6.61E-08
HORVU.MOREX.r3.2HG0102150	ABC transporter G family member	0.41573393	0.05431958	0.85994059	2.90E-07
HORVU.MOREX.r3.3HG0331080	Plant cadmium resistance protein	0.4152397	0.054636	0.94411484	4.25E-11
HORVU.MOREX.r3.5HG0474720	Short-chain dehydrogenase/reductase family protein	0.41508762	0.05473365	0.9262271	6.34E-10
HORVU.MOREX.r3.5HG0475900	NBS-LRR disease resistance protein, putative, expressed	0.41465542	0.05501189	0.84948761	5.70E-07
HORVU.MOREX.r3.2HG0193660	Ubiquinol oxidase	0.41446208	0.05513671	0.8623046	2.47E-07
HORVU.MOREX.r3.3HG0282640	ABC transporter B family protein	0.41401874	0.05542376	0.80495158	6.25E-06
HORVU.MOREX.r3.5HG0536960	Protein kinase	0.41387645	0.05551613	0.93563541	1.69E-10
HORVU.MOREX.r3.3HG0220030	UDP-glucosyl transferase 85A7	0.41185617	0.05684043	0.80044182	7.70E-06

HORVU.MOREX.r3.4HG0386880	Peptidase M50B-like protein	0.40964154	0.05831983	0.94123997	6.94E-11
HORVU.MOREX.r3.1HG0054950	Chitinase	0.40884191	0.05886117	0.91797904	1.77E-09
HORVU.MOREX.r3.2HG0190470	Argonaute family protein	0.40867914	0.05897184	0.89038521	2.85E-08
HORVU.MOREX.r3.1HG0050830	F-box family protein	0.40782397	0.05955587	0.90376907	8.22E-09
HORVU.MOREX.r3.7HG0657240	Cysteine-rich receptor-kinase-like protein	0.5909319	0.00377976	0.81680595	3.52E-06
HORVU.MOREX.r3.5HG0530780	Serine/threonine-protein kinase	0.50418254	0.01672732	0.8183565	3.26E-06
HORVU.MOREX.r3.5HG0509540	PRA1 family protein	0.47864823	0.02422547	0.81211889	4.44E-06
HORVU.MOREX.r3.3HG0283720	Regulator of Vps4 activity in the MVB pathway protein	0.47767863	0.02455578	0.83260778	1.53E-06
HORVU.MOREX.r3.7HG0644350	Lung seven transmembrane receptor family protein	0.46678799	0.02851882	0.90229315	9.51E-09
HORVU.MOREX.r3.6HG0600160	Ethylene-responsive transcription factor	0.45323485	0.03414205	0.91549925	2.36E-09
HORVU.MOREX.r3.3HG0254600	NAC domain-containing protein	0.44835573	0.03636774	0.8706276	1.38E-07
HORVU.MOREX.r3.7HG0726170	Mitogen-activated protein kinase	0.44351846	0.03868531	0.90265612	9.18E-09

HORVU.MOREX.r3.1HG0000610	Charged multivesicular body protein 5	0.44030797	0.04028627	0.80164392	7.29E-06
HORVU.MOREX.r3.1HG0083600	Lipid phosphate phosphatase-like protein	0.43696694	0.04200685	0.92281918	9.82E-10
HORVU.MOREX.r3.3HG0305600	Protein EFR3-like protein	0.42927966	0.04618337	0.81354464	4.14E-06
HORVU.MOREX.r3.3HG0266070	Vesicle transport v-SNARE family protein	0.42290745	0.04988388	0.85851984	3.19E-07
HORVU.MOREX.r3.5HG0521040	Vesicle-associated membrane protein, putative	0.41500204	0.05478866	0.90892117	4.85E-09
HORVU.MOREX.r3.3HG0288830	MACPF domain protein	0.41480269	0.05491696	0.83833578	1.11E-06
HORVU.MOREX.r3.3HG0322040	Receptor protein kinase, putative	0.41311666	0.05601136	0.90617931	6.44E-09
HORVU.MOREX.r3.3HG0270050	Kinase family protein	0.58841208	0.00396959	0.84363757	8.13E-07
HORVU.MOREX.r3.5HG0440170	ATP-dependent zinc metalloprotease FtsH	0.58687533	0.00408923	0.86843877	1.61E-07
HORVU.MOREX.r3.5HG0530810	Ammonium transporter	0.57385193	0.00522952	0.94318929	4.99E-11
HORVU.MOREX.r3.7HG0642300	DUF674 family protein	0.54486843	0.00873661	0.80405796	6.52E-06
HORVU.MOREX.r3.7HG0650260	WRKY family transcription factor	0.5398155	0.00951132	0.85397164	4.29E-07

HORVU.MOREX.r3.5HG0494070	Purple acid phosphatase	0.51673902	0.01380121	0.86098248	2.71E-07
HORVU.MOREX.r3.3HG0281310	Receptor-like kinase	0.50910935	0.01552458	0.91013333	4.26E-09
HORVU.MOREX.r3.4HG0380710	Calmodulin	0.48427774	0.02237711	0.81797471	3.32E-06
HORVU.MOREX.r3.7HG0708000	O-glucosyltransferase rumi	0.47118868	0.02686046	0.83678812	1.21E-06
HORVU.MOREX.r3.4HG0412300	Receptor-like protein kinase, putative	0.4612237	0.03073094	0.8893456	3.12E-08
HORVU.MOREX.r3.2HG0100660	Transmembrane protein 115	0.44970703	0.03574022	0.83852459	1.10E-06
HORVU.MOREX.r3.7HG0723420	Eukaryotic aspartyl protease family protein	0.44564428	0.03765297	0.85964656	2.96E-07
HORVU.MOREX.r3.5HG0534720	Receptor-like protein kinase	0.44215424	0.03935939	0.84586994	7.11E-07
HORVU.MOREX.r3.3HG0281270	Kinase family protein	0.43867947	0.0411179	0.87555736	9.53E-08
HORVU.MOREX.r3.5HG0463440	NAC domain protein,	0.43511668	0.04298407	0.80973913	4.98E-06
HORVU.MOREX.r3.4HG0332100	Leucine-rich repeat receptor-like protein kinase family protein	0.43503266	0.04302886	0.92048337	1.31E-09
HORVU.MOREX.r3.5HG0512600	Peroxidase	0.4305044	0.04549722	0.8135109	4.15E-06
HORVU.MOREX.r3.4HG0353220	Coiled-coil domain-containing protein SCD2	0.42406239	0.04919672	0.86397781	2.21E-07

HORVU.MOREX.r3.4HG0333410	Thaumatin like protein	0.42369844	0.04941247	0.90883938	4.89E-09
HORVU.MOREX.r3.3HG0230520	NBS-LRR disease resistance protein	0.42031432	0.05145377	0.80718122	5.63E-06
HORVU.MOREX.r3.7HG0677510	Glycosyltransferase	0.42027273	0.05147925	0.82642431	2.14E-06
HORVU.MOREX.r3.4HG0382620	Argininosuccinate lyase	0.41903313	0.05224334	0.8572629	3.47E-07
HORVU.MOREX.r3.4HG0403960	Lectin receptor kinase	0.41851176	0.0525673	0.83391203	1.42E-06
HORVU.MOREX.r3.7HG0683800	Exocyst complex component, putative	0.4105913	0.05768181	0.94159304	6.54E-11
HORVU.MOREX.r3.6HG0567460	Protein-O-fucosyltransferase 1	0.57490061	0.00512886	0.82069431	2.89E-06
HORVU.MOREX.r3.2HG0147980	ADP-ribosylation factor	0.5633393	0.00633272	0.86540261	2.00E-07
HORVU.MOREX.r3.2HG0187560	ORMDL family protein-like	0.52063157	0.01298393	0.9472438	2.42E-11
HORVU.MOREX.r3.1HG0069880	Endoplasmic reticulum-Golgi intermediate compartment protein 3	0.4808726	0.02348108	0.87791309	7.95E-08
HORVU.MOREX.r3.6HG0625870	Proteasome maturation factor UMP1 family protein	0.4615116	0.03061325	0.84593055	7.09E-07
HORVU.MOREX.r3.5HG0509950	Transmembrane emp24 domain-containing protein	0.45231803	0.03455187	0.84997652	5.53E-07

HORVU.MOREX.r3.1HG0063340	B-cell receptor-associated 31-like	0.44089748	0.03998848	0.86012252	2.87E-07
HORVU.MOREX.r3.4HG0385780	Mitogen-activated protein kinase	0.44005005	0.0404171	0.86024942	2.84E-07
HORVU.MOREX.r3.2HG0161020	3-oxoacyl-reductase	0.4336605	0.04376553	0.85494645	4.03E-07
HORVU.MOREX.r3.5HG0513020	Transmembrane protein 147	0.43339875	0.04390717	0.82192292	2.71E-06
HORVU.MOREX.r3.7HG0684240	Transmembrane emp24 domain-containing protein	0.43135849	0.04502344	0.88372108	5.01E-08
HORVU.MOREX.r3.5HG0469790	Serine/threonine protein phosphatase 2a regulatory subunit A, putative	0.43026677	0.04562973	0.80739441	5.57E-06
HORVU.MOREX.r3.4HG0335500	Proteasome subunit beta type	0.42928003	0.04618316	0.80531128	6.15E-06
HORVU.MOREX.r3.6HG0629510	Acetyl-coenzyme A synthetase	0.42657132	0.04772921	0.91787096	1.79E-09
HORVU.MOREX.r3.3HG0303190	UDP-glucuronate decarboxylase protein 1	0.42548224	0.048362	0.82099156	2.85E-06
HORVU.MOREX.r3.4HG0412360	PLAC8 family protein	0.42529978	0.04846865	0.96584061	3.39E-13

HORVU.MOREX.r3.2HG0192180	26S proteasome non-ATPase regulatory subunit 1	0.42355634	0.0494969	0.85150348	5.02E-07
HORVU.MOREX.r3.5HG0454880	Chaperone protein dnaJ	0.42236082	0.05021169	0.80106916	7.49E-06
HORVU.MOREX.r3.5HG0459320	Peptidylprolyl isomerase	0.42039712	0.05140306	0.83497128	1.34E-06
HORVU.MOREX.r3.2HG0100320	Cullin-associated NEDD8-dissociated protein 1	0.41518063	0.05467391	0.80203713	7.16E-06
HORVU.MOREX.r3.4HG0360670	RING/FYVE/PHD zinc finger superfamily protein	0.41253196	0.05639478	0.89956019	1.24E-08
HORVU.MOREX.r3.1HG0067040	NAC domain protein,	0.41163438	0.05698727	0.83986825	1.02E-06
HORVU.MOREX.r3.2HG0163690	Kinase family protein	0.4109646	0.05743251	0.89526256	1.85E-08
HORVU.MOREX.r3.1HG0078730	Signal recognition particle 54 kDa protein	0.40927705	0.05856611	0.81396839	4.05E-06

Supplementary Table S4.2: Gene Overrepresentation Test for Upregulated DEGs in SALMON Module

GO biological process complete	*H. vulgare* subsp. vulgare - REFLIST (34193)	Client Text Box Input (822)	Client Text Box Input (expected)	Client Text Box Input (over/under)	Fold Enrichment	P-value	FDR
regulation of defense response to bacterium (GO:1900424)	2	2	0.05	+	41.6	5.77E-04	3.47E-02
intra-Golgi vesicle-mediated transport (GO:0006891)	6	6	0.14	+	27.73	4.79E-06	6.31E-04
cell surface receptor protein serine/threonine kinase signaling pathway (GO:0007178)	5	3	0.12	+	24.96	1.34E-04	1.04E-02
defense response to oomycetes (GO:0002229)	7	3	0.17	+	17.83	4.51E-04	2.81E-02
tryptophan biosynthetic process (GO:0000162)	14	5	0.34	+	0.34	1.33E-05	1.57E-03
retrograde vesicle-mediated transport, Golgi to endoplasmic reticulum (GO:0006890)	20	5	0.48	+	10.4	9.11E-05	8.01E-03
defense response to bacterium (GO:0042742)	29	7	0.7	+	10.4	4.45E-06	6.10E-04

chitin catabolic process (GO:0006032)	17	4	0.41	+	9.79	6.15E-04	3.51E-02
calcium ion transmembrane transport (GO:0070588)	28	6	0.67	+	8.91	4.54E-05	4.72E-03
cell wall macromolecule catabolic process (GO:0016998)	25	5	0.6	+	8.32	'2.83E-04	'2.02E-02
recognition of pollen (GO:0048544)	125	20	3.01	+	6.66	'2.21E-11	'6.91E-09
defense response to fungus (GO:0050832)	59	7	1.42	+	4.94	'5.21E-04	'3.19E-02
carbohydrate transport (GO:0008643)	83	8	2	+	4.01	'8.69E-04	'4.52E-02
protein transport (GO:0015031)	422	26	10.14	+	2.56	'1.42E-05	'1.57E-03

Supplementary Table S4.3: Gene Overrepresentation Test for Upregulated DEGs in BLACK Module

GO biological process complete	*H. vulgare* subsp. vulgare - REFLIST (34193)	Client Text Box Input (822)	Client Text Box Input (expected)	Client Text Box Input (over/under)	Fold Enrichment	P-value	FDR
negative regulation of translational frameshifting (GO:2001125)	2	2	0.04	+	53.51	3.49E-04	1.04E-02
oxaloacetate transport (GO:0015729)	2	2	0.04	+	53.51	3.49E-04	1.03E-02
thiosulfate transport (GO:0015709)	2	2	0.04	+	53.51	3.49E-04	1.02E-02
box H/ACA sno(s)RNA 3'-end processing (GO:0000495)	2	2	0.04	+	53.51	3.49E-04	1.01E-02
box C/D sno(s)RNA 3'-end processing (GO:0000494)	2	2	0.04	+	53.51	3.49E-04	1.01E-02
S-adenosylmethionine cycle (GO:0033353)	2	2	0.04	+	53.51	3.49E-04	1.00E-02
snRNA modification (GO:0040031)	3	3	0.06	+	53.51	6.50E-06	3.56E-04
UDP-glucuronate biosynthetic process (GO:0006065)	4	3	0.07	+	40.13	2.56E-05	1.19E-03

glycosaminoglycan biosynthetic process (GO:0006024)	4	3	0.07	+	40.13	2.56E-05	1.18E-03
protein folding in endoplasmic reticulum (GO:0034975)	3	2	0.06	+	35.67	1.03E-03	2.73E-02
succinate transmembrane transport (GO:0071422)	3	2	0.06	+	35.67	1.03E-03	2.71E-02
seryl-tRNA aminoacylation (GO:0006434)	3	2	0.06	+	35.67	1.03E-03	2.69E-02
UDP-D-xylose biosynthetic process (GO:0033320)	5	3	0.09	+	32.11	6.32E-05	2.52E-03
ribosomal small subunit assembly (GO:0000028)	5	3	0.09	+	32.11	6.32E-05	2.48E-03
malate transmembrane transport (GO:0071423)	4	2	0.07	+	26.76	2.04E-03	4.93E-02
protein secretion (GO:0009306)	4	2	0.07	+	26.76	2.04E-03	4.85E-02
UDP-L-arabinose metabolic process (GO:0033356)	4	2	0.07	+	26.76	2.04E-03	4.80E-02
eukaryotic translation initiation factor 4F complex assembly (GO:0097010)	4	2	0.07	+	26.76	2.04E-03	4.74E-02

endonucleolytic cleavage of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA) (GO:0000479)	11	5	0.21	+	24.32	9.45E-07	6.31E-05
chorismate biosynthetic process (GO:0009423)	9	4	0.17	+	23.78	1.41E-05	7.11E-04

Appendix B

FIRST REPORT OF RICE BACTERIAL LEAF BLIGHT DISEASE CAUSED BY *PANTOEA ANANATIS* IN THE UNITED STATES[3]

In August 2021, bacterial leaf blight-like symptoms were observed on 14 out of 570 rice genotypes (*Oryza sativa*) in research field plots of global rice germplasm grown in Arkansas (Fig. B1. A & B). The disease was characterized by spreading lesions on leaves, panicle sterility and reduced yield in highly susceptible, mature rice germplasm. No spread of disease to nearby plants was observed. Isolations were performed at Colorado State University, where soakates from symptomatic leaves were spread onto nutrient agar. After 72 h at 28°C, uniform, distinct, yellow-colored bacterial colonies were observed. To screen for the presence of common rice bacterial pathogens, PCR amplification directly from colonies or from DNA isolated from symptomatic field-collected leaves was performed. Primers specific for *Xanthomonas oryzae* pvs. *oryzae* and *oryzicola* (Lang et al., 2010), *Burkholderia glumae* (Echeverri-Rico et al., 2021), and *Pseudomonas fuscovaginae* (Ash et al., 2014) did not amplify indicating these organisms were not present. Sequencing of 16S rRNA gene (Weisburg et al., 1991) amplicons suggested the bacteria belonged to the genera *Pantoea* and *Sphingomonas* (NCBI accession no. OP683332 and OP683333, respectively). Amplicons resulting from primers specific to the *gyrB* gene region

[3]Published as Luna, E., Lang, J., McClung, A., Wamishe, Y., Jia, Y., & Leach, J. E. (2023). First report of rice bacterial leaf blight disease caused by *Pantoea ananatis* in the United States. Plant Disease, 107(7), 2214.

Contributions by E Luna: Performed and designed experiments; wrote manuscript

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of *P. ananatis* (Kini et al., 2021) were sequenced and the fragment was compared to the *P. ananatis* PA13 reference genome using a BLAST analysis. One candidate (AR358) showed 100% identity with the *P. ananatis gyrB* region. Primers specific for *Sphingomonas* sp. (Bangratz et al., 2020) confirmed the second candidate (AR359) as a *Sphingomonas* sp. The identity of *P. ananatis* was confirmed by the Plant Pathogen Confirmatory Diagnostics Laboratory (Beltsville, MD, USA).

To determine pathogenicity, leaves from 7-day-old seedlings of rice (*Oryza sativa*) cultivar Kitaake were scissor-clip inoculated (Kauffman et al., 1973) with four different treatments and compared to control leaves inoculated with sterile water. Treatments for the experiment consisted of bacterial suspensions (10^8 CFU/ml) of the two candidate organisms, *P. ananatis* (strain AR358) or *Sphingomonas* sp. (strain AR359), individually or in a 1:1 ratio of *P. ananatis*:*Sphingomonas* sp., or soakate from infected field tissue. Lesions similar to those observed in the field were only detected on leaves inoculated with *P. ananatis* or infected field tissue soakate at 7-days post-inoculation (Fig. B1. C). Bacteria were recovered from the leaves of the artificially inoculated seedlings from three treatments (*P. ananatis*, *P. ananatis*:*Sphingomonas* sp. and soakate from the infected field tissue) and were determined to be *P. ananatis* based on colony morphology, amplification of 16s rRNA, and *gyrB* sequence data. Our results confirm the pathogenicity of *P. ananatis* to rice and fulfill Koch's postulates. *P. ananatis* was also recovered from several similarly diseased rice breeding lines at the University of Arkansas System Division of Agriculture Rice Research and Extension Center. We conclude that *P. ananatis* is the causal pathogen for leaf blight-like symptoms observed in the global rice cultivars grown in Arkansas. *P. ananatis* was previously reported as a pathogen on rice in

several rice growing regions, including China (Yu et al., 2021), India (Reshma et al., 2022), and Africa (Kini et al., 2017), however, this is the first report of *P. ananatis* as a pathogen of rice in the United States.

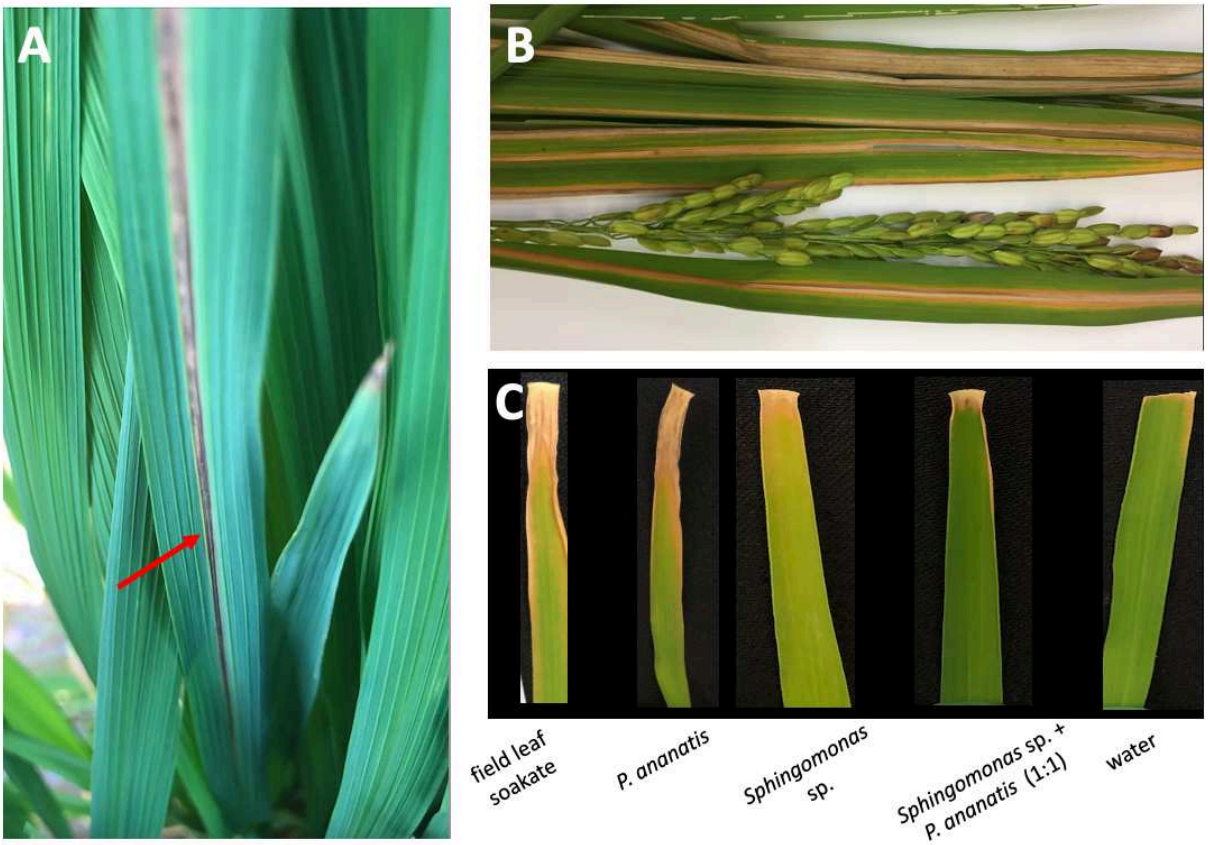

Fig. B1: (A) Early (indicated by red arrow) and (b) late season symptoms of rice leaves from Arkansas fields. (C) Representative photos from Koch's postulates experiment. Rice leaves were scissor-clip inoculated with *P. ananatis* or *Sphingomonas* sp.*,* individually or in a 1:1 ratio of *P. ananatis*:*Sphingomonas* sp., or with soakate from infected field tissue.

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