Horizontal transmission and host plant interactions in the Lagriinae-Burkholderia-symbiosis

Masterarbeit
zur Erlangung des akademischen Grades
Master of Science (M.Sc.)

vorgelegt von
Paul Gaube
Jena, 2016

Friedrich-Schiller-Universität Jena
Biologisch-Pharmazeutische Fakultät
Institut für Mikrobiologie

Die vorliegende Arbeit wurde am Max-Planck-Institut für Chemische Ökologie, Abteilung Insektensymbiosen unter der Betreuung von Prof. Dr. Martin Kaltenpoth angefertigt.

Erstgutachter. Prof. Dr. Martin Kaltenpoth
Max-Planck-Forschungsgruppe Insektensymbiosen,
Max-Planck-Institut für Chemische Ökologie Jena,

Zweitgutachter. Prof. Dr. Erika Kothe
Institut für Mikrobiologie, Mikrobielle Kommunikation,
Friedrich-Schiller-Universität Jena

„A World is not an ideology nor a scientific institution, nor is it even a system of ideologies; rather, it is a structure of unconscious relations and symbiotic processes."
William Irwin Thompson

Contents

1. Summary **1**

2. Zusammenfassung **2**

3. Introduction **4**

3.1 Insect-microbe symbiosis 4

3.1.1 Insect-*Burkholderia* symbioses 5

3.1.2 Lagria-*Burkholderia* symbiosis 5

3.2 Symbiont transmission modes 7

3.2.1 Transmission of *Burkholderia* in insects 7

3.2.2 Transmission of insect-associated microbes via plants 7

3.2.3 Transmission routes of *Burkholderia* symbionts in *Lagria* 8

3.3 Biology and occurrence of *Burkholderia gladioli* 9

3.4 Plant defensive systems against phytopathogens 11

3.5 Objectives 12

4. Material and Methods **13**

4.1 Beetle collection and symbiotic organ dissection 13

4.2 Calibration curve for measuring bacterial cell quantity using optical density 14

4.3 Nucleic acid extraction 15

4.4 PCR amplification, agarose gel electrophoresis and sequence analysis 15

4.5 cDNA Analysis 17

4.5.1 Reverse Transcription 17

4.5.2 Quantitative Real Time Polymerase Chain Reaction 17

4.6 Fluorescence in situ hybridization 18

4.7 Horizontal transmission experiments 19

4.7.1 Transfer of bacterial symbionts from beetles to leaf litter 19

4.7.2 Acquisition of bacterial symbionts from leaf litter by larvae 20

4.8 Plant fitness effect upon *B. gladioli* infection 21

4.9 Cotyledon assay 23

4.9.1 Bacterial strains 23

4.9.2 Inoculation of cotyledons and detection of phytoalexins 23

5. Results **25**

5.1. *In vitro* phenotypic variation of *B.gladioli* strain A 25

5.2 Localization of *Burkholderia* in the symbiotic organs of *L. villosa* 25
5.2.1. Adult females 25
5.2.2. Larvae 26
5.3 Horizontal transmission of the *Burkholderia* symbionts 28
5.3.1 Transmission of symbionts from *Lagria villosa* adult beetles to leaves 28
5.3.2 Acquisition of *Burkholderia* from leaf litter by aposymbionts 28
5.4 Interaction between *B. gladioli* and soybean plants 31
5.4.1 Systemic plant infection by *Burkholderia* symbiont of *L. villosa* 31
5.4.2 Impact on plant fitness upon infection with the symbiotic *B. gladioli* ... 34
5.4.3 Phytoalexin response in soybean upon *Burkholderia* infection 37
6. Discussion 40
6.1 Phenotypic variation of symbiotic *B. gladioli* strain A 40
6.2 Horizontal transmission of *Burkholderia* symbionts 41
6.2.1 Transmission of symbionts from *L. villosa* adult beetles to leaves 41
6.2.2 Acquisition of symbionts and their translocation to the symbiont housing organs 42
6.3 Soybean plant-*Burkholderia* interaction 45
6.3.1 Effect of *Burkholderia* on soybean plants 45
6.3.2 Defensive reaction of the host plant 48
6.4 Tripartite interaction between the insect, the plant and the bacteria 50
6.5 Conclusions and perspectives 51
References 53
List of abbreviations 68
Supplemental Material 70
Danksagung 74
Selbstständigkeitserklärung 75

1. Summary

Microbes can establish symbiotic associations with various eukaryotic hosts and can have different effects on their hosts, ranging from beneficial to detrimental. There are several examples of three way interactions between plants, microbes and insects, where microbial symbionts are transmitted between their different hosts and affect the physiology or ecology of the organisms involved. On the one hand, plant pathogenic microbes can be transmitted by insects, on the other hand plants can mediate the horizontal transfer of mutualistic microorganisms among their host insects. However, a system, in which a plant associated microbe can be both, detrimental to a plant and beneficial to an insect by independent mechanisms, has to our knowledge not been shown before.

In the present Master thesis the symbiotic association between *Lagria villosa* beetles and their *Burkholderia* mutualists has been characterized under the aspect of plant-mediated horizontal transmission. Furthermore, it has been investigated how the bacterial symbionts interact with soybean plants, on which the beetles naturally occur. The symbionts are transmitted vertically (from mother to offspring*)* on the surface of eggs, where they inhibit the growth of pathogenic fungi. However, phylogenetic analyses imply that the lagriid symbionts, which are very close related to plant pathogenic *B. gladioli*, can also be transmitted horizontally. This study provides the first experimental evidence that *Lagria villosa* beetles can acquire mutualistic *B. gladioli* from the environment and integrate them into symbiont carrying organs. Exactly 85% (17 of 20 individuals) of tested aposymbiotic female individuals, that were exposed until they reached adulthood to leaves containing *Burkholderia*, harbored symbionts within the accessory glands of the reproductive system. However, how this process of symbiont integration is regulated by the insect hosts is not known until now. The insects are also able to transmit their symbionts to leaves of soybean plants and to dry leaves of pea, raps and soybean plants, where they can persist. It was shown that symbiotic *B. gladioli* have the potential to infect soybean plants systemically and affect the fitness of soybean plants. The infected plants suffered from more pronounced chlorosis in older leaves and produced significantly less seeds, but also started flowering earlier and grew higher. Overall, the symbiotic *B. gladioli* strain isolated from *L. villosa* had no drastic but weak

pathogenic effects on soybean plants. Contrastingly, cotyledon assays showed a defensive response by means of glyceollin production against symbiotic bacteria from *L. villosa*, indicating that *B. gladioli* strain A is recognized by the plant early stages as a pathogen, whereas the *B. gladioli* strain G cultured from the European species *L. hirta* did not affect a response in the cotyledon assay.
In conclusion, this interkingdom host-symbiont interaction can be a useful system to understand how plant pathogenic bacteria evolved to insect mutualists. This opens questions that are not only relevant for ecological and evolutionary aspects, but can also be of interest to economic issues in agriculture.

2. Zusammenfassung

Mikroorgansimen können symbiotische Beziehungen mit verschiedenartigen eukaryotischen Wirten eingehen. Sie können unterschiedliche Effekte auf ihre Wirte haben, welche von vorteilhaft bis nachteilig reichen. Es gibt etliche Beispiele von Drei-Wege-Interaktionen zwischen Pflanzen, Mikroben und Insekten, in denen mikrobielle Symbionten zwischen verschiedenen Wirten übertragen werden und die Physiologie oder Ökologie der involvierten Organismen beeinflusst wird. Einerseits können pflanzenpathogene Mikroben durch Insekten übertragen werden, anderseits können Pflanzen den horizontalen Transfer von mutualistischen Mikroorganismen zwischen ihren Wirtsinsekten vermitteln. Trotzdem, ein System, in welchem eine pflanzenassoziierte Mikrobe beides sein kann, schädlich für eine Pflanze und nützlich für ein Insekt durch unabhängige Mechanismen, wurde unseres Wissens, vorher noch nicht gezeigt.
In der vorliegenden Masterarbeit wurde die symbiotische Gemeinschaft zwischen *Lagria villosa*-Käfern und ihren *Burkholderia*-Mutualisten unter dem Aspekt von pflanzenvermittelter horizontaler Transmission charakterisiert. Des Weiteren wurde untersucht, wie die bakteriellen Symbionten mit der Sojapflanze interagieren, auf welcher die Käfer natürlicherweise vorkommen. Die Symbionten werden auf der Eioberfläche vertikal weitergegeben (von der Mutter zu dem Nachwuchs), wobei diese das Wachstum von pathogenen Pilzen inhibieren. Allerdings implizieren phylogenetische Analysen, dass die Symbionten der Lagriiden, welche sehr nah verwandt mit pflanzenpathogenen *B. gladioli* sind, auch horizontal übertragen

werden können. Diese Studie liefert den ersten experimentellen Beweis, dass *Lagria villosa*-Käfer sich mutualistische *B. gladioli* aus der Umwelt aneignen und diese in die Symbionten tragenden Organe integrieren können. Exakt 85% (17 von 20 Individuen) der getesteten aposymbiotischen weiblichen Individuen, welche bis zum Erreichen der Imagophase Blättern, die Burkholderien enthielten, exponiert waren, beherbergten Symbionten in den akzessorischen Drüsen des Reproduktionssystems. Jedoch ist bis jetzt noch nicht bekannt, wie dieser Prozess der Symbiontenintegration durch die Wirtsinsekten reguliert ist. Die Insekten sind auch dazu fähig, ihre Symbionten auf Sojapflanzen und trockenen Erbsen-, Raps und Sojablättern zu übertragen, wo diese fortbestehen. Es wurde gezeigt, dass symbiotische *B. gladioli* das Potenzial haben Sojapflanzen systematisch zu infizieren und die Fitness von Sojapflanzen zu beeinträchtigen. Die infizierten Pflanzen erlitten eine höher ausgeprägte Chlorosis in älteren Blättern und produzierten signifikant weniger Samen, aber blühten auch früher und wuchsen höher. Insgesamt hatte der symbiotische *B. gladioli* Stamm, welcher von *L. villosa* isoliert wurde, keine drastischen, aber schwache pathogene Auswirkungen auf die Sojapflanzen. Dagegen zeigten Untersuchungen mit Kotyledonen eine Verteidigungsreaktion gegen symbiotische Bakterien von *L. villosa*, mittels Glyceollinproduktion. Das weist darauf hin, dass der *B. gladioli* Stamm A von frühen Pflanzenstadien als Pathogen erkannt wurde, wohingegen der *B. gladioli* Stamm G, welcher von der europäischen Spezies *L. hirta* kultiviert wurde, keine Verteidigungsreaktion auslöste.

Abschließend kann gesagt werden, dass „Interkingdom“-Wirts-Symbiont-Beziehungen ein wertvolles System darstellen können, um zu verstehen wie pflanzenpathogene Bakterien zu Mutualisten in Insekten evolvieren. Das eröffnet Fragen, die nicht nur relevant für ökologische und evolutionäre Aspekt sind, sondern auch von ökonomischem Interesse in der Landwirtschaft sein können.

3. Introduction

3.1 Insect-microbe symbiosis

Symbiosis, as defined by de Bary in 1879, is a broad term that describes the living together of dissimilar organisms in an intimate association with each other. Symbiotic interactions among insects and microorganisms are very common in nature and range from antagonistic (parasitism), in which one partner imposes a fitness cost upon the other, to neutral for one of the partners (commensalism) and up to beneficial for all partners (mutualism). The high abundance, diversity and success of insects in occupying various ecological niches are thought to be influenced by their mutualistic symbionts (Kikuchi, 2009; Moran et al., 2008; Smith, 1989). Symbiotic microorganisms can be localized in different parts of the insect body, often intracellularly in bacteriocytes or extracellularly in the gut lumen or within specialized structures associated to the digestive system (Bourtzis & Miller, 2009; Buchner, 1965). They can also be found in the hemocoel (Moran, Russell, et al., 2005) or associated to the fat body (Dale et al., 2006), in neural ganglia (Hypsa, 1993), in the hemolymph (Hypsa & Dale, 1997), in the reproductive system and related accessory glands (Stammer, 1929) as well as in antennal glands (Kaltenpoth et al., 2005).

Microbial symbionts can play a crucial role in detoxification or digestion of complex compounds and essential amino acid- or vitamin provision (Baumann et al., 2006; Breznak & Brune, 1994; Douglas, 2009). Defense is another important role in a number of mutualistic relationships between insects and microorganisms (Brownlie & Johnson, 2009; Currie et al., 1999; Flórez et al., 2015; Kaltenpoth et al., 2005; Kellner, 2002; Oliver & Moran, 2009; Scott et al., 2008; Um et al., 2013). Additionally, it is coming into focus of an increasing number of studies since new antimicrobial drugs are more and more required. As Actinobacteria, Proteobacteria are especially common defensive symbionts protecting against pathogens, parasitoids or predators (Flórez et al., 2015). It is known that bacteria from this group defend Coleoptera (Cardoza et al., 2006; Kellner, 2002; Piel, 2002), Diptera (Teixeira et al., 2008) and Heteroptera (Becerra et al., 2015; Moran, Degnan, et al., 2005; Oliver et al., 2003; Scarborough et al., 2005) that is potentially facilitated by the production of defensive toxins.

3.1.1 Insect-*Burkholderia* symbioses

Burkholderia have been found in a number of insect groups, although a symbiotic role has only been demonstrated in a number of heteropteran species. In bugs, of the family Largidae and the superfamilies Lygaeoidea and Coreoidea, *Burkholderia* spp. symbionts are harbored within specialized crypts associated to the gut (J. R. Garcia et al., 2014; Kikuchi et al., 2011a; Kikuchi et al., 2005; Sudakaran et al., 2015). In the broad-headed bean bug *Riptortus pedestris*, the *Burkholderia* symbionts have been shown to directly increase host fitness (Kikuchi et al., 2007) aid in insecticide detoxification (Kikuchi et al., 2012), and enhance the innate immune system of the host (J. K. Kim et al., 2015). In other bug species the removal of *Burkholderia* symbionts also results in retarded development and decreased survival of the host (Bourtzis & Miller, 2009; Olivier-Espejel et al., 2011).

Furthermore, there were *Burkholderia* found in other insect families. Nevertheless, neither they have been demonstrated as (real) symbionts nor is there conclusive evidence that they play a functional role for their hosts. *Burkholderia* sp. with the potential to produce antifungal compounds was found in colonies of the leaf-cutting ant *Atta sexdens*. It was suggested that these bacteria could inhibit the growth of pathogenic fungi on the host and the fungus gardens, although evidence for a consistent association and a defensive role is lacking (Santos et al., 2004). In another ant species the *Burkholderia* found in the gut might be involved in the oxidative recycling of nitrogen-rich metabolites (van Borm et al., 2002). *Burkholderia* were also found within the digestive tract of a ground beetle, in which a role in the digestion of complex compounds was suggested yet not experimentally supported (Lundgren et al., 2007).

3.1.2 Lagria-*Burkholderia* symbiosis

A novel defensive symbiosis was recently found in *Lagria villosa* beetles (Coleoptera: Tenebrionidae, Lagriinae), in which *Burkholderia gladioli* (ß-Proteobacteria) produce compounds that defend eggs and possibly also larvae against fungal pathogens (Flórez et al., in preparation). The symbionts are located in a pair of accessory glands associated to the female reproductive system (Figure 1A). As described in the closely related *Lagria hirta*, the bacterial symbionts are transmitted vertically from the mother to the offspring by smearing the eggs with a bacteria-

containing secretion during oviposition. The symbionts enter the egg shell and migrate into dorsal invaginations of the embryo (Figure 1B) (Stammer, 1929). This localization of the symbiotic structures in the larval stage is to our knowledge unique among described insect symbiosis. It is still unknown how the symbionts migrate from the larval dorsal compartments to the adult accessory glands. Although, other bacterial groups are also recognized in DNA extracted from the adult symbiotic organs, *B. gladioli* is the most abundant and consistent taxon (Flórez et al., in preparation). Sub-species level analyses of high-troughput sequencing data suggest the presence of at least three different *Burkholderia gladioli* strains, of which only one was culturable so far (Flórez & Kaltenpoth, in preparation). The latter will be referred to as *B. gladioli* strain A from now on. Rearing *L. villosa* beetles under laboratory conditions resulted in occasional loss of the *Burkholderia* symbionts or a change in strain profiles after one or more generations (Flórez & Kaltenpoth, in preparation).

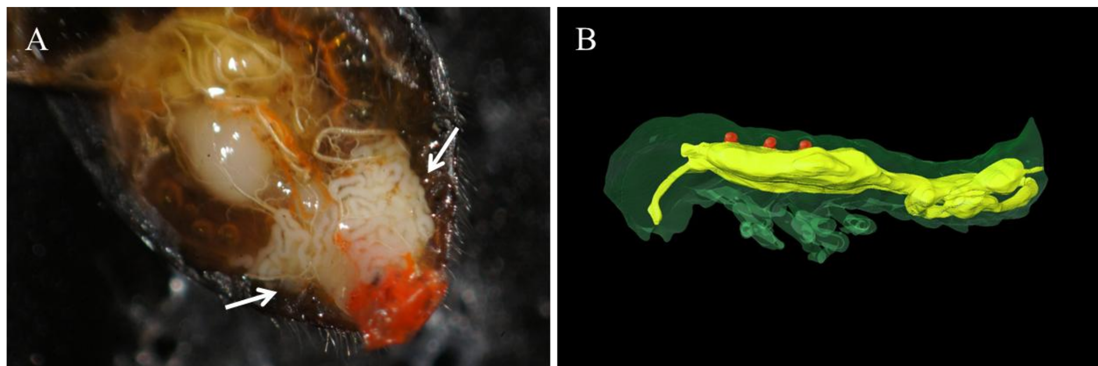

Figure 1. *Lagria villosa* symbionts are localized within (**A**) accessory glands connected to the female reproductive system (arrows) and (**B**) in three dorsal compartments represented in red in a 3D reconstruction of a larva. Picture (**A**) by L. Flórez and 3D reconstruction (**B**) by B. Weiss.

Lagria villosa Fabr. is originally from Africa and invasive in South America, predominantly in Brazil (Edwards, 1977; Pacheco et al., 1976; Spilman, 1978). This species feeds on leaves of various plants including soybean (Montero et al., 2002; Villas Boas, 1982), beans, coffee (Pacheco, 1978), cotton (M. A. Garcia & Pierozzi Junior, 1982), potato (Azeredo & Rodrigues Cassino, 2004), rape (Montero et al., 2010) and strawberries (Setti de Liz et al., 2009).

3.2 Symbiont transmission modes

Mechanisms for symbiont transmission range from strictly vertical (from parents to offspring) to strictly horizontal (from conspecifics or the environment). However, there is often a combination of both mechanisms, called mixed-mode transmission, which can occur within one or more generations (Ebert, 2013). Vertical transmitted symbionts are usually aligned with their host in terms of survival and reproduction, and thus selective pressures on beneficial symbionts are stronger under these conditions. Horizontal transmission implies symbiont acquisition from other hosts or often from natural environment; therefore plants can play an important role as a source or temporary reservoir of symbiotic bacteria. In systems with mixed transmission mode, a failure in one mechanism for symbiont acquisition can be compensated by the other, thus reducing the probability of symbiont loss by the host.

3.2.1 Transmission of *Burkholderia* in insects

Transmission events of *Burkholderia* have been exclusively however rarely reported in heteropteran insects. In oriental chinch bugs (family: Blissidae) vertical transmission occurs at an estimated rate of 30%, in combination with environmental acquisition (Itoh et al., 2014).

In contrast to vertical transmission events of *Burkholderia* symbionts, horizontal transmission has been described in more detail. Previous studies by Kikuchi and coauthors revealed that the broad-headed bean bug *Riptortus pedestris* (synonym *R. clavatus)* acquires symbionts from the environment every generation, likely from the soil of soybean plants (Kikuchi et al., 2007). Like other bug species, *R. pedestris also* harbors symbionts in crypts, predominatly *Burkholderia* which improves hosts fitness. It was shown that the symbionts are taken up from the environment during the second and third nymphal instars (Kikuchi et al., 2011b). While infections with larger bacterial cell titers lead to higher efficiency, an infection dose with even 80 cells resulted in 50% successful colonization (Kikuchi & Yumoto, 2013).

3.2.2 Transmission of insect-associated microbes via plants

Plants can mediate horizontal transmission of insect symbionts as it is reported in the whitefly-*Rickettsia* symbiosis (Caspi-Fluger et al., 2012). The secondary symbionts

were transmitted from the insect host *Bemisia tabaci* to basil, cotton and black nightshade plants, where they colonized phloem cells and spread throughout the plant. Consequently, *Rickettsia*-free whiteflies were reinfected via feeding on the phloem sap.

Furthermore, it was shown that interspecific horizontal transmission of *Cardinium* symbionts can occur through plants tissue (Gonella et al., 2015). The symbionts were transferred through the salivary glands of the monophagous leafhopper *Scaphoideus titanus* to the phloem of grapevine leaves and subsequently acquired by feeding on the same diet by two other leafhopper species, *Macrosteles quadripunctulatus* and *Empoasca vitis*. The horizontal transmission route via inner parts of the plant is also frequently used by plant pathogenic bacteria that are spread by insect vectors (Nadarasah & Stavrinides, 2011).

However, plants are not only involved actively as a source or sink in symbiont transmission; they can also serve as a passive surface. In the plataspid stinkbug *Megacopta punctatissima*, obligate symbionts belonging to the γ-proteobacteria, are deposited in capsules next to the eggs on the leaf surface, and newborn nymphs ingest the capsules, by which vertical transmission occurs (Fukatsu & Hosokawa, 2002).

3.2.3 Transmission routes of *Burkholderia* symbionts in *Lagria*

Burkholderia symbionts are transmitted vertically in *Lagria villosa* by smearing of the eggs surface at the process of laying eggs (Stammer, 1929). However, a phylogenetic analysis of 16S rDNA sequences from lagriid-associated *B. gladioli* showed that these do not form a single (monophyletic) clade that distinguishes them from closely related environmental relatives (Flórez et al., in preparation). Rather, the insect symbionts are randomly distributed within several plant pathogens of *B. gladioli*, a pattern typical for horizontal transmission (Figure 2). This also suggests that individual symbionts are likely not specialized to single hosts and that environmental acquisition of symbionts takes place. The random distribution of insect symbiotic and plant pathogenic bacteria within the *B. gladioli* cluster suggests that there were exchanges of symbionts between lagriid beetles or with the environment. In theory, there were several events of symbiont acquisition from environment, possibly facilitated by feeding or contact with host plants.

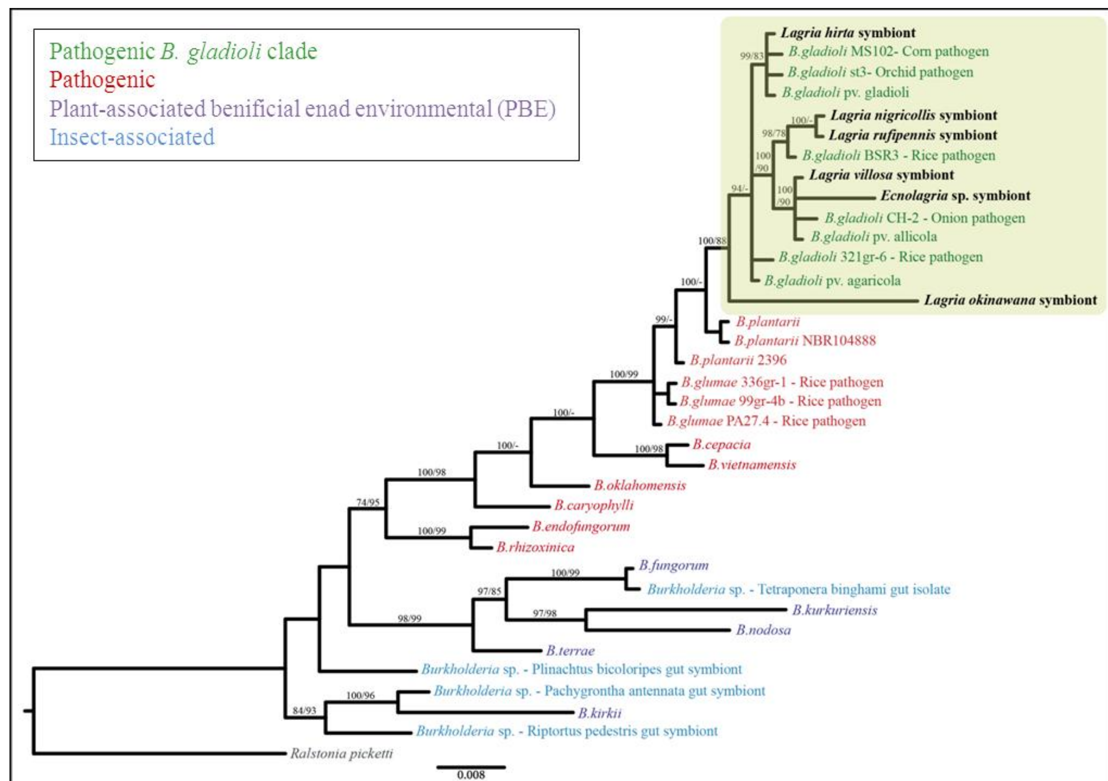

Figure 2. Phylogenetic reconstruction based on Bayesian and approximately-maximum-likelihood algorithms of selected *Burkholderia* using partial 16S rRNA gene sequences (1148bp) shows the scattered placement of lagriid-associated *Burkholderia* within a cluster of plant-pathogenic *B. gladioli*. Posterior probabilities and local support values above 0.7 are reported at the nodes (modified from Flórez et al., in preparation).

3.3 Biology and occurrence of *Burkholderia gladioli*

Burkholderia are aerobic, motile, non-fermenting, gram-negative and rod-shaped bacteria which have been assigned to a separate genus since 1992 (Yabuuchi et al.); previously belonging to the RNA homology group II *Pseudomonas*. W. H. Burkholder firstly described *B. cepacia* in 1942 as a plant pathogen causing disease in onion (Burkholder, 1950). This species is ubiquitous as many other species within this genus. *Burkholderia* can be found as endophytes in plants, in soil, water, in the rhizosphere, also involved in plant nodulation, in various animals or fungi and in the last 20 years some species have emerged as severe human pathogens (Coenye & Vandamme, 2003).

Like other *Burkholderia* species, *B. gladioli* exhibits a relatively broad range of lifestyles. Originally, it was identified as a phytopathogen of gladiolus (Severini, 1913) and further pathovar strains are known today as pathogens of plants (in

gladiolus, iris, freesia, onions, tulips and rice) and fungi (button mushrooms) (Chowdhury & Heinemann, 2006; C. J. Lee et al., 2005; Saddler, 1994a, 1994b; Ura et al., 2006). Disease symptoms vary from scabbing of foliar parts, to spotting of necrotic lesions or chlorosis, soft decay over entire leaves and rotting of storage tissues. However, some strains were also found as non-pathogenic endophytes in coffee (Vega et al., 2005), rice (Jha et al., 2015), sweet corn (Ettinger et al., 2015; McInroy & Kloepper, 1995) and soybean (Kuklinsky-Sobral et al., 2005). On account this, it can be likely that *L. villosa* beetles, which can be often found in soybean and coffee plantations in Brazil, transmit their *B. gladioli* symbionts to these plants.

Furthermore, *B. gladioli*'s antifungal activity against a broad spectrum of phytopathogenic fungi has become increasingly relevant in agriculture (Elshafie et al., 2012; Jha et al., 2015; Yan et al., 2015). Although primarily a phytopathogen *B. gladioli* has become more frequent as a cause of pulmonary infection in cystic fibrosis and other immune-compromised patients (Coenye & LiPuma, 2003; Kennedy et al., 2005; J. P. Ross et al., 1995; Segonds et al., 2009; Simpson et al., 1994). The spectrum of disease locations and symptoms expanded within the last years to bacteremia (Shin et al., 1997), osteomyelitis (Boyanton et al., 2005), otitis media (Foley et al., 2004) peritonitis (Tong et al., 2013), and sepsis (Dursun et al., 2012). *B. gladioli* show resistance against several antibiotics, especially ß-lactams and tetracyclins (Angus et al., 2014). However, they show sensitivity to aminoglucozides, chinolones and imipenem (Stoyanova et al., 2007).

Genomic studies in several *B. gladioli* strains have revealed the presence of multiple virulence factors like capsules (EPS) (Andolfi et al., 2008; Angus et al., 2014; Furuya et al., 1997b), fimbriae and flagella (Angus et al., 2014), glycoproteins (Seo et al., 2015), lipopolysaccharides (Furuya et al., 1997b), pili (Angus et al., 2014), quorum sensing (Angus et al., 2014; Seo et al., 2015), cellulases, lipases, polygalacturonases and proteases (Seo et al., 2015), and it is known to produce some toxic compounds (Moebius et al., 2012; C. Ross et al., 2014), e.g. toxoflavin that can also act as a phytotoxin (Furuya et al., 1997a). Toxoflavin is produced by almost all strains of *B. gladioli*, including the human pathovar *cocovenenans* as well (Iiyama et al., 1998; Seo et al., 2015; Vandamme et al., 1960). This strain produces another highly toxic compound, bonkrekik acid that is produced in a fermented soybean-coconut product (tempe bonkrèk) and also has plant pathogenic potential (Moebius et

al., 2012; Passam, 1975). Nevertheless, little is known about the principles of molecular interaction between the bacteria and an infected plant (but see Lee et al., 2015). Virulence factors like cell wall-degrading enzymes, proteases, toxins and other effectors to defeat host cells are secreted by different secretion systems in *B.gladioli*. They are essential to produce surface structures for adhesion and for aggregation, as well as for cell motility (Chang et al., 2014). In the well-studied rice pathogen strain BSR3, gene clusters involved in secretion systems of type II, III, IV and VI were located (Angus et al., 2014; Fory et al., 2014; Seo et al., 2015). Type II secretion system is involved in the secretion of protease, chitinase and plant cell wall-degrading enzymes (Chang et al., 2014; Seo et al., 2015). The type III secretion system is essential as it secretes effector proteins and toxins, like toxoflavin, via injectisomes directly into host cells (Chang et al., 2014; Seo et al., 2015). It has been shown that toxoflavin plays a major role in symptomatic disease development in rice (Jeong et al., 2003). Also Type IV and VI secretion systems are involved in secreting effector proteins and virulence factors (Chang et al., 2014). Nevertheless, it is still unknown whether *Burkholderia gladioli* symbionts harbored by *Lagria villosa* retain their phytopathogenic potential. Phylogenetically they are very close related (above 96% similarity at the 16S rRNA gene) to *B. gladioli* plant pathogens causing onion soft rot (C. J. Lee et al., 2005) and to strain BSR3, a phytopathogen causing panicle blight in rice seeds (Nandakumar et al., 2007).

3.4 Plant defensive systems against phytopathogens

In general, there are two plant-pathogen interactions, a compatible interaction (host susceptible) and an incompatible interaction (host resistant). Compatible interactions are characterized by extensive multiplication and possible systemic spread of pathogens within the host plant, which leads to ultimate symptoms and death. A hallmark of incompatible plant-pathogen interactions is the resistance of non-host plants triggered by pathogen-specific elicitor recognition by the plant. There is limited or no pathogen multiplication and usually no macroscopically visible symptoms. Nevertheless, at high inoculum levels (> 5×10^{6} CFU ml^{-1}) a rapid necrosis can occur, often induced by hypersensitive response as a defense mechanism analogous to the innate immune system in animals (Tampakaki et al., 2009)

Besides reactive oxygen species (ROS), phytoalexins play an important role in many incompatible plant-pathogen interactions (Paxton, 1981). They are antimicrobial active compounds produced and accumulated in plants after attack by microorganisms, insects or nematodes as well as abiotic stresses. Glyceollin is the major phytoalexin produced in soybean and it occurs as a series of five isomers. These different isomers are produced in different parts and life stages of the plant (Paxton, 1995). Glyceollin production occurs in both types of plant-pathogen interactions, in incompatible interactions together with hypersensitive response and in compatible interactions with the absence of hypersensitive response and a lower accumulation of glyceollins (Bruegger & Keen, 1979; Mohr & Cahill, 2001). Several microbial race-specific elicitors have been characterized so far. Betaglucans from fungal cell walls, which can also be found in bacterial cell walls, have been described as the most active. These elicitors also trigger the production of the isoflavones daidzein and genistein. Daidzein is a precursor of glyceollin, while genistein is not directly involved in the glyceollin synthesis. Both have also antimicrobial activity, but less than glyceollin, and they have antiestrogenic properties as well (Nwachukwu et al., 2013).

3.5 Objectives

The present study is focused on the tripartite interaction between a lagriid beetle, its bacterial symbionts and soybean plants. The main question was if insect-symbiotic *Burkholderia* bacteria could be transmitted via the plant and how this affects the plant host.

The first objective was to investigate whether *Lagria* beetles can acquire and maintain *Burkholderia* from leaves and vice versa, as a potential way of horizontal transmission. Therefore it was also examined if the bacterial transfer from host plant to beetle could result in the occupation of the female symbiotic organ, as occurs in naturally infected beetles.

The second objective of this study was to investigate the plant infection potential of symbiotic *Burkholderia* and if this occurs locally or systemically. Furthermore, we assessed whether there is a pathogenic effect on the host plant upon infection.

4. Material and Methods

4.1 Beetle collection and symbiotic organ dissection

Lagria villosa and *hirta* species were reared under greenhouse conditions with light-dark cycle of 16 to 8 hour light-dark cycle at 24 ± 2 °C (humidity 50-60%) and 14 to 10 hours at 21 ± 2 °C (humidity 45-55%), respectively. *Lagria villosa* beetles were collected between January and February 2015 (SISBIO authorization Nr. 45742-1, CNPq process n° 01300.004320/2014-21) from Corumbatai, Itaju and Sao Carlos regions (Sao Paulo state, Brazil). They were fed with fresh pea, rape and soybean leaves. *Lagria hirta* imagines were collected from Ammerbach, Jena, Gerrmany (50.90094, 11.54950) and fed with fresh birch, maple, linden and wayfarer leaves. The larvae received the respective diet as dry leaves. Autoclaved tap water was supplied in 50 ml falcon tubes covered with sterile cotton. *Lagria hirta* larvae were kept at 4 °C for at least one month to induce diapause, while *L. villosa* larvae remained at the temperatures mentioned above for their entire life cycle.

A single *Burkholderia gladioli* symbiotic strain had been previously isolated from each beetle species *L. villosa* and *L. hirta* (strain A and strain G, respectively). However, there is evidence that multiple symbiotic strains are present in these beetles (Flórez & Kaltenpoth, in preparation). In an attempt to isolate additional *Burkholderia* strains from the insects, the symbiont-containing accessory glands of three female *Lagria villosa* and *Lagria hirta* were dissected and suspended in 100 µL PBS. To achieve a potentially higher spectrum of isolated bacteria, three different media, namely KB, Grace and SF900 with supplements (fatty acid 1 µg ml^{-1}, GABA 5 mg mL^{-1}, N-acetylglucosamine 0.1 mg mL^{-1}, spermidine 25 µg mL^{-1} and urea 10 mg mL^{-1}) were used. Cultivation resulted in an average bacteria concentration of about 1.9×10^6 CFUs in *L. hirta*. Unfortunately, cultivation of bacteria from dissected accessory glands of *L. villosa* resulted in non-cultivable bacteria. DNA was isolated from 35 isolated colonies, followed by 16S rRNA gene amplification using the primer pair Burk3.1fwd and BKH1434 (Table 1) and subsequent Sanger sequencing (Sanger et al., 1977). Sequencing resulted in at least 14 high quality sequences of target gene fragments. These were aligned against consensus sequences of cultivated *B. gladioli* strain A (*L. villosa)* and strain G (*L. hirta*), which are highly similar at the 16S rRNA gene. Strain A and strain G differ only in five single base

mutations at fragment length of 1311 bp (99.62% identity). Comparison of the obtained sequences against reference strains resulted in 100% sequence identity with *B. gladioli* strain G. Thus, *Burkholderia gladioli* strain A and strain G were used to infect *L. villosa and L. hirta* larvae, respectively. These isolated strains were also used for the plant infection experiments.

4.2 Calibration curve for measuring bacterial cell quantity using optical density

An OD/CFU-calibration was performed for the three *B. gladioli* strains used in subsequent experiments. Bacterial cultures were grown for 48 h under constant shaking (200 rpm) at 30 °C. *B. gladioli* strain A and G were grown in KB liquid medium, whereas LB broth was used for *B. gladioli* pv. *cocovenenans*. Dilution series between 10^0 to 10^{-7} were prepared in triplicate, and optical density was measured at 600 nm with an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). Four different dilutions were plated on KB and LB medium and CFUs were counted after 2 days. Only counts between 20 - 200 per plate were considered for the final calibration curve (Figure 3).

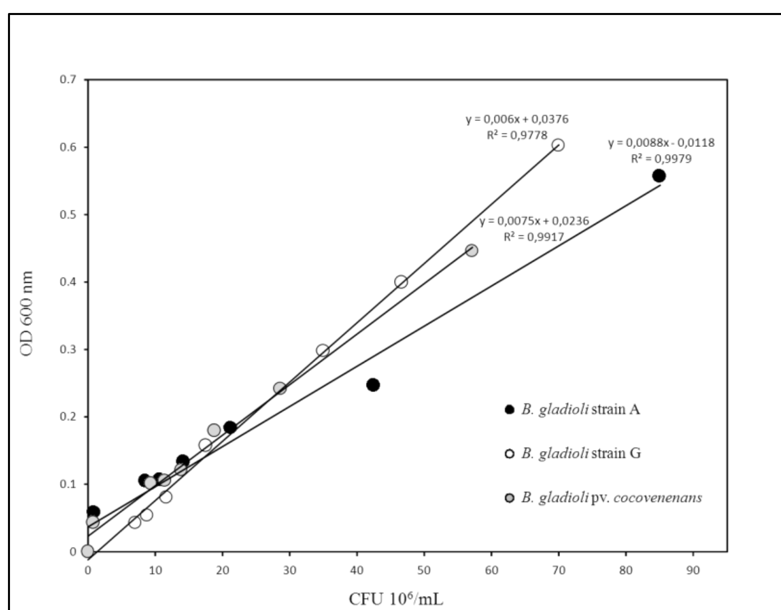

Figure 3. Calibration curve of OD_{600} in relation to CFU of symbiotic *Burkholderia* strain A (cultivated from *L. villosa*), strain G (cultivated from *L. hirta*) and pathovar *cocovenenans*. Linear regression was performed on the mean of three replicated measurements, respectively. Appropriate equations and R-square values are displayed.

4.3 Nucleic acid extraction

Bacterial cells, as well as insect and plant tissue were crushed in Eppendorf tubes with liquid nitrogen using blunt pipette tips and subjected to DNA and RNA extraction with the MasterPure™ Complete DNA and RNA Purification Kit (Illumina, Madison, USA) according to the manufacturer´s instructions. Samples for RNA extraction were separated after the Protein precipitation step, followed by the RNA purification protocol. Centrifugation steps were performed at 4°C and DNA and RNA were resuspended in Low TE-buffer (10 mM Tris/HCL, 0.1 mM EDTA). All solutions used for RNA extraction were prepared in DEPC (Diethylpyrocarbonate) treated H_2O and RNaseZap Decontamination Solution (Thermo Fisher Scientific, Waltham, USA) was used in order to avoid RNase contamination.

4.4 PCR amplification, agarose gel electrophoresis and sequence analysis

The presence of *Burkholderia* was tested by diagnostic PCR using the *Burkholderia* specific primers listed in Table 1. Primer pairs Burk3.1fwd and BKH1434Rw were used to sequence a larger fragment of the 16S rRNA gene (1291 bp) and primers gla-FW and gla-RV were used for sequencing the gyraseB gene (about 770 bp). Primers targeting the coleoptera 18S rRNA gene were used to test the success and quality of DNA extractions by assessing whether insect host DNA is still present, particularly in individuals that had died early before extraction. Primers targeting *B. gladioli* general and strain specific 16S rRNA gene were used to confirm strain presence in the transmission or infection experiments. PCR amplifications were performed on a T-Professional-Gradient Thermocycler (Biometra, Göttingen, Germany) and carried out in a 12.5 µL reaction volume containing 5.36 µL of Millipore H_2O, 1xPCR buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 2.5 mM $MgCl_2$, 2 mM dNTPs, 10 pmol/µL of each forward and reverse primer, 0.5 U of *Taq* DNA polymerase (Roboklon, Berlin, Germany) and 1 µL template. A H_2O (Millipore) sample was used as a negative control in order to assess for possible contamination or unspecific amplification.

Table 1. Specific primers used for diagnostic and quantitative PCR.

Primer name	Sequence (5'-3')	Target gene	Reference
18S ai	CCTGAGAAACGGCT ACCACATC	Coleoptera 18S rRNA	Shull 2001
18S bi	GAGTCTCGTTCGTT ATCGGA	Coleoptera 18S rRNA	Shull 2001
BKH1434Rw	TGCGGTTAGRCTAS CYACT	*Burkholderia* 16S rRNA	Opelt et al. 2007
Burk16S_1_F	GTTGGCCGATGGCT GATT	*Burkholderia* 16S rRNA	Flórez et al., in preparation
Burk16S_1_R	AAGTGCTTTACAAC CCGAAGG	*Burkholderia* 16S rRNA	Flórez et al., in preparation
Burk16S_StA-G_F	CTGAGGGCTAATAT CCTTCGGGG	*Burkholderia* 16S rRNA	Flórez et al., unpublished
Burk16S_StB_F	TTGAAGGCTAATAT CCTTCAAGA	*Burkholderia* 16S rRNA	Flórez et al., unpublished
Burk16S_StF_F	CTTTGGGCTAATAC CCTGAGGGG	*Burkholderia* 16S rRNA	Flórez et al., unpublished
Burk3.1fwd	CCCKGCRAAAGCCG GAT	*Burkholderia* 16S rRNA	modified from Salles et al. 2002
Burk3.1Rev	TRCCATACTCTAGC TTGC	*Burkholderia* 16S rRNA	modified from Salles et al. 2002
gla-FW	CTGCGCCTGGTGGT GAAG	gyrase B of *B. gladioli*	Maeda et al. 2006
gla-RV	CCGTCCCGCTGCGG AATA	gyrase B of *B. gladioli*	Maeda et al. 2006

PCR cycle parameters were as follows: an initial denaturation step at 94 °C for 4 min, followed by 32 cycles of 94 °C for 40 s, an annealing step at 65 °C for 60 s and 72 °C for 60s. A final extension step of 72 °C for 4 min was performed and the samples were stored at 4 °C before gel electrophoresis. PCR parameters used for obtaining PCR products for sequencing analysis were performed with 35 cycles at 94 °C for 40 s (denaturation), 62 C for 40 s (annealing) and 72 °C for 90 s (extension). Final denaturation and final extension steps were conducted as described previously. Gel electrophoresis of all PCR products was carried out in 1.5% agarose gels stained with GelRed^{TM} (Biotium, Hayward, USA). Documentation and processing of gel pictures was performed with GeneSnap image acquisition software (Version 7.09.06, Syngene, Cambridge, UK). PCR products were purified with the innuPREP PCRpure kit according to the manufacturer´s protocol (Analytik, Jena, Germany). DNA concentrations were measured using a NanoDropTM1000 spectrophotometer (Peqlab, Erlangen, Germany).

Amplicons of *gyrase B* and 16S rRNA genes were sequenced bidirectionally with a capillary sequencer (Sanger et al., 1977) at the Entomology Department of the Max Planck Institute for Chemical Ecology using the same primers as for the PCR amplification. The sequences were analyzed and edited with Geneious (version 6.0.5,

Biomatters, Auckland, New Zealand) and aligned against reference sequences of *B. gladioli* symbiotic strains from *Lagria hirta* and *Lagria villosa*.

4.5 cDNA Analysis

4.5.1 Reverse Transcription

RNA from both transmission and plant infection experiments were reverse transcribed to cDNA for quantification and detection of living *Burkholderia* in tissue samples. Reverse transcription was carried out with the QuantiTect® Reverse Trancription kit (Qiagen, Hilden, Germany) and single steps were performed on ice following the manufacturer´s protocol. The gDNA was firstly removed from each sample by adding 2 µL of gDNA Wipeout Buffer (7x) to 1 µg of RNA filled up with RNase- free water to 12 µL. Subsequently, the reaction was incubated at 42 °C for 3 min. The reaction mix was then mixed with 1 L Quantiscript Reverse Transcriptase, 4 L RT Buffer (5x) and 1 µL of RT Primer Mix containing random primers. The reaction was incubated firstly for 15min at 42 °C and again for 5min at 95°C to inactivate the reverse transcription.

4.5.2 Quantitative Real Time Polymerase Chain Reaction

Quantitative PCR was conducted in a RotorGene®-Q cycler (Qiagen, Hilden, Germany). The total reaction volume of 25 µL contained 12.5 µL SYBR Green Mix (Qiagen), 6.5 µL water (Merck), 2.5 µL (10mM) of each primer and 1 µL cDNA template, obtained as previously described. The qPCR procedure consisted of an initial denaturation step at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s, primer annealing at 65 °C for 30 s and elongation at 72 °C for 20 s. A melting curve was performed after each cycle step with a temperature gradient from 60 °C to 99 °C within 4.25 min. DNA standards for quantification of the target fragments were obtained previously by PCR amplification and product recovery using the innuPREP Gel Extraction Kit (Analytik). Purified DNA concentration was measured using a NanoDropTM1000 spectrophotometer (Peqlab). Standards were used as a tenfold dilution series ranging from 10^{-1} to 10^{-8} ng µL^{-1}.

4.6 Fluorescence in situ hybridization

To visualize *Burkholderia* symbionts within larval dorsal structures or within the accessory glands of female reproductive systems, fluorescence in situ hybridization (FISH) was performed on cross-sections of *L. villosa*. Samples were individually fixed in 70 % ethanol, dehydrated in acetone and then embedded in Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer´s instructions. Sections of 5-8 µm thickness were prepared with a diamond knife on a Microm HM 355 S microtome (Thermo Scientific, Waltham, USA). Positive controls containing *Burkholderia* cells were prepared in 70% EtOH and dried at around 50 °C. FISH was performed as described by Kaltenpoth et al. (2005) using a hybridization temperature of 55°C for 90 min. Hybridization was conducted in a moist chamber with different fluorescent bacterial probes (50 ng μL^{-1}) (Table 2), depending on the experiment, dissolved in hybridization buffer (HB, 900 mM NaCl, 0.01% SDS, 20 mM and Tris/HCl pH 8). Then, the slides were washed with washing buffer (5 mM EDTA, 100 mM NaCl, 0.1% SDS and 20 mM Tris/HCl pH 8) followed by an incubation step with WB for 20 min at 55 °C. Subsequently, the slides were rinsed with distilled water and incubated for another 20 min at room temperature. Finally, slides were air dried in darkness and mounted with VECTASHIELD (Biozol Diagnostica, Eching, Germany). Slides were examined under an AxioImager Z1 fluorescent microscope (Zeis, Oberkochen, Germany) and pictures were taken with an AxioCam MRm colour camera (Zeiss) and processed with AxioVision software (Lite Edition 4.8.1, Zeiss).

Furthermore whole mount FISH was carried out on thinly sliced leaf and stem tissue of *G. max* to locate *B. gladioli* within different plant parts. Plant material was kept in Carnoy’s fixative (ethanol: chloroform: acetic acid, 6:3:1) until further use. Samples were washed for 2 h in 100% ethanol and bleached in 6% H_2O_2 (in EtOH) for around two weeks. The bleaching solution was changed every third day. Afterwards samples were washed as in 100% ethanol (3 x 2 h), in 70% EtOH (2 x 1 h). and in PBS-Tx buffer (4 x 0.5 h). Hybridization was performed overnight (24 h) in Eppendorf tubes containing 270 ul HB, 15 µL of each probe (50 ng μL^{-1}) and 3 µl DAPI (0.5 mg ml^{-1}) for host cell counterstaining. Several washing steps followed (2 x 2 h in WB and 2 x 30 min in distilled H_2O). Samples were incubated in distilled H_2O and small

amount of VECTASHIELD for 2 h at room temperature. Before the microscopical analysis samples were transferred to a slide and covered with VECTASHIELD.

Table 2. Fluorescent probes used for FISH. The general eubacterial probe EUB338 was used for FISH carried out on insect tissue, the chloroplast non-matching probe EUB784 was used for FISH performance on plant tissue.

Probe	Sequence (5'-3')	Direction	5'-modification	Target gene	Reference
Burk_16S	TGCGGTTAGACT AGCCACT	rev.	Cy3 or Cy5	Burkholderia 16S rRNA	modified from Opelt et al. 2007
EUB338	GCTGCCTCCCGT AGGAGT	rev.	Cy3 or Cy5	Eubacteria 16S rRNA	modified from Amann et al. 1990
EUB338_ reversed	ACTCCTACGGGA GGCAGC	fwd.	Cy3	negative control probe	modified from Amann et al. 1990
EUB784	TGGACTACCAGG GTATCTAATCC	rev.	Cy3 or Cy5	most Eubacteria 16S rRNA	designed byMartin Kaltenpoth

4.7 Horizontal transmission experiments

4.7.1 Transfer of bacterial symbionts from beetles to leaf litter

Field-collected *L. villosa* beetles from Brazil were reared under greenhouse conditions as described above in twelve plastic boxes, each containing groups of five to six individuals with at least one female per box. One piece of both fresh and dry leaves of *G. max*, *P. sativum* and *B. napus* was placed inside larval boxes every second day. Ten additional boxes without *L. villosa* beetles were used as controls following the same procedure. After 30 days of exposure, dry leaves were recovered. To test whether bacteria can be transmitted and survive on leaf litter, complete leaf material was homogenized followed by DNA and RNA extraction of a defined amount using the MasterPure™ Complete DNA and RNA Purification Kit (Illumina, Madison). PCR reactions were performed in 6.5 µL reactions using primers Burk3.1fwd and BKH1434Rw (Table 1) and PCR program as described for this pimer pair in section 4.4 and a gel electrophoresis was carried out with the PCR products as described above. Primers Burk16S_1_F and Burk16S_1_R (Table 1) amplifying a 211 bp fragment were used for qPCR analysis and the corresponding statistical analysis was performed via SPSS (version 17.0; SPSS Inc., Chicago, USA).

4.7.2 Acquisition of bacterial symbionts from leaf litter by larvae

To obtain aposymbiotic individuals, *Lagria* eggs were submerged in 95% ethanol for 5 min and rinsed thoroughly with autoclaved water. Subsequently, they were sterilized in 12% NaClO for 30 s and rinsed again with autoclaved water. Additionally, a reinfected “natural” (R-Nat) treatment was generated to monitor separately the success of reinfection on the egg stage. To obtain these R-Nat individuals, eggs were infected after surface sterilization with a PBS suspension in which the natural microbiota of untreated eggs was previously recovered. Sterile PBS served as a control for aposymbiotic eggs. The individuals that had been surface sterilized at the egg stage and therefore expected to be aposymbiotic, were used for the acquisition experiment of *B. gladioli* symbionts. To test if these were in fact symbiont-free, a diagnostic PCR with *Burkholderia* specific primers (Burk3.1fwd and BKH1434Rw, Table 1) was performed, shortly before the experiment started, on a subset of six individuals, confirming the absence of *Burkholderia*. Fresh and dried leaves of *G. max*, *P. sativum* and *B. napus* were inoculated with a 20 µL suspension containing 10^6 CFUs of symbiotic *B. gladioli* strain A by distributing the suspension on the leaves with a paintbrush. As a control, leaves were smeared with sterilized tap in a comparable manner. Aposymbiotic *Lagria villosa* larvae at second to third instar were exposed to leaves for 41±7 days under 14:10 h light:dark photoperiod conditions. Temperature and humidity were monitored during the experiment. Previously inoculated leaves were placed inside larvae boxes every second day and sterilized drinking water was replaced once a week. *L. villosa* individuals were collected shortly after they reached adulthood, surface sterilized in 70% ethanol and their sex was determined by dissecting the beetles with fine forceps and microscissors under a binocular microscope in a glass petri dish on wax cooled with ice. The paired symbiotic structures were separated carefully from the gut and the rest of the body. One of the two structures was kept at -80°C for testing the presence of bacterial symbionts by extracting DNA and RNA, with diagnostic and quantitative PCR, respectively. The other half was kept in 70% ethanol for visualization of *Burkholderia* using fluorescence in situ hybridization. Diagnostic PCR was performed with the strain A specific primer pair Burk16S_StA-G_F and Burk3.1Rev (191 bp fragment) as well as with primers targeting strain B specific 208 bp regions (Burk16S_StB_F and Burk3.1Rev) (Table 1).

A symbiont acquisition experiment was also performed with single reared *Lagria hirta* larvae, however the experiment failed, because all individuals died before they reached adulthood.

4.8 Plant fitness effect upon *B. gladioli* infection

Glycine max [L.] Merr. cv. Alligator seedlings (N.L.Chrestensen Erfurter Samen- und Pflanzenzucht GmbH) were grown for 28 days in 6 cm diameter pots at around 25°C under greenhouse conditions. After seven days, plants with comparable height were transferred to individual pots for the experiment. After reaching second stage, one leaflet of each trifoliate was wounded in the middle area using a robotic device that mimics insect herbivory (Mithofer et al., 2005) to make a circular 0.5 cm diameter wound (Figure 4). Symbiotic *Burkholderia L. villosa* strain A cells were previously cultured in KB liquid media under constant shaking conditions (200 rpm) at 30°C for 48 hours. This culture was diluted to 10^5 CFUs μL^{-1} and resuspended in sterilized tap water. 18 Plants were inoculated with 10 µL bacteria solution on the wounded area of the trifoliate leaves. The same volume of sterilized water was used as control inoculum for other 18 plants. The plants were grown for 38 days post infection with light and dark cycle at 16 h daylight conditions. Height, time of first flowering and time of first leaf fall were monitored about every second day. Chlorophyll contents of the two infected leaves of each plant were measured in triplicate on the same spot by using a chlorophyll meter SPAD-502Plus (Konica Minolta, Marunouchi, Japan). The chlorophyll content of treated older leaves (first trifoliate leaflet) and treated younger leaves (second trifoliate leaflet) were compared between infected and control plants for the duration of the experiment. At the end, plants without roots and separated from the seeds were dried at 70°C for 24 h to determine the total dry weight, as well as seed dry weight and seed output. Pieces of leaves and stem of different plant tissues were taken for visualization of infiltrated bacteria by fluorescence in situ hybridization. To detect *Burkholderia* presence in different parts of the plants, pieces of leaf tissues from (i) the wounded area, (ii) a distant area on the wounded leaf and (iii) a not wounded leaflet of a younger leaf, were taken for DNA and RNA extraction (Figure 4). A diagnostic PCR for the presence of strain A was conducted with the primer pair Burk16S_StA-G_F and Burk3.1Rev (Table 1) of a 191 bp fragment and quantitative PCR was carried out for

Burkholderia specific 16SrRNA gene on the corresponding cDNA with the same primers used for diagnostic PCR. Statistical analyses were performed using SPSS (version 17.0; SPSS Inc., Chicago, USA) and RStudio (version 0.98.1103, RStudio Inc., Boston, USA) was used for generalized linear mixed model analysis using individual plant as random factor, and either chlorophyll content and time or height and time as fixed factors.

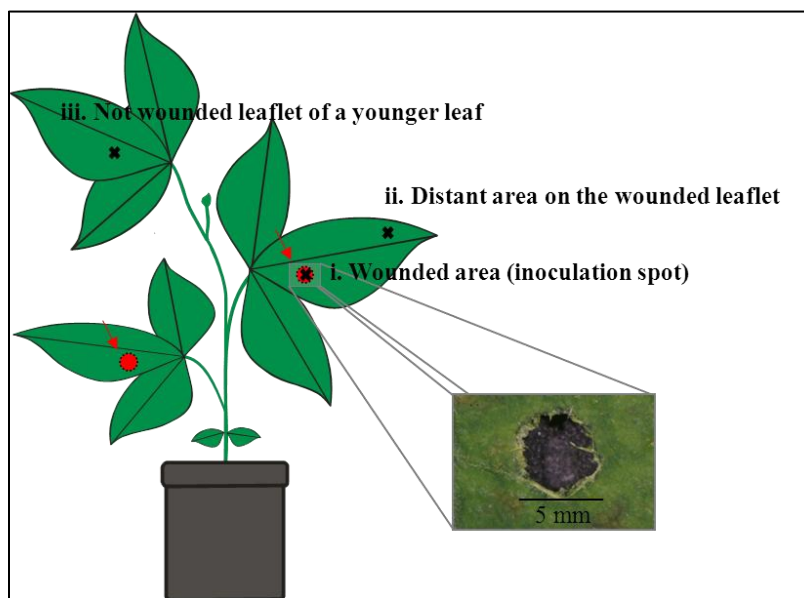

Figure 4. Schematic representation of plant infection-fitness assay setup, illustrating two wounding spots on first and second trifoliate leaf (bacterial infection spots, red arrows) and leaf pieces taken from different parts of the soybean plant and used for quantitative PCR detection of *Burkholderia gladioli* (black crosses).

In addition, a further plant infection experiment with a *G. max* cultivar from Colombia (Semillas Panorama) was performed to test different infection conditions. Bacterial strains given in Table 3 were used to screen a higher spectrum of potential plant pathogenicity effects. Two leaves were wounded with a needle on the same spot as described before and inoculated by syringe pressure infiltration with 20µl bacterial solutions, each containing 10^6 cells (Chakravarthy et al., 2009). Sterile tap water was used as a negative control. The plants were grown under the same conditions and monitored as described in the previous experiment, yet no nucleic acid extraction was carried out.

4.9 Cotyledon assay

4.9.1 Bacterial strains

King's B (KB) medium was used for the growth of bacteria except *strain B. gladioli* pv. *cocovenenans* wherefore LB medium was used. The bacterial cultures were grown from glycerol stocks for 48 h at 30°C and continuous shaking at 200 rpm. A fraction of symbiotic *B. gladioli* strain A cells were inactivated by suspending them in 70% ethanol for 5 min. Bacterial cultures were centrifuged (10.000 rpm, 2 min) and resuspended in sterile filtered tap water at a final concentration of 10^6 CFUs 50 μL^{-1}. The optical density (600 nm) of *E. coli* K-12 corresponding to the required cell concentration was calculated based on information provided by the manufacturer (http://www.genomics.agilent.com/biocalculators/calcOD-Bacterial.jsp). That is, a concentration of $8x10^5$ CFUs μL^{-1} corresponding to OD_{600} = 1. All bacterial strains and respective treatments used in the cotyledon bioassay are listed in Table 3.

Table 3. Treatments and bacterial strains used for cotyledon assay and 2nd plant infection assay.

Leaves infiltrated with	Source
sterile tap H_20	autoclaved and sterile filtrated tap water
ß-glucan	cell wall of *Phytophtora sojae*
E.coli K12	Agilent Technologies, Santa Clara, USA
dead *B. gladioli* strain A	culltivated from *L. villosa*
B. gladioli strain A	cultivated from *L. villosa*
B. gladioli strain G	cultivated from *L. hirta*
B. gladioli pv. *cocovenenans*	DSMZ 11318

4.9.2 Inoculation of cotyledons and detection of phytoalexins

Cotyledons were obtained from 5-day old *Glycine max* L. (Semillas Panorama, Colombia) plantlets. Cotyledons were initially washed with distilled water and placed in 10% NaClO for 5 min, followed by another round of washing with distilled water for 20 minutes. The cotyledon bioassay was performed as described by Bruegger and Keen (1979) with minor modifications. Cotyledons were cut with a straight razor blade on the lower side of each cotyledon. Groups of ten cotyledons were used with three replicates for each treatment. The Cotyledons were placed on moistened filter paper and 50 µL of either bacterial solution (10^6 CFUs 50 μL^{-1}), sterile tap H_2O (negative control) or ß-glucan as an elicitor (positive control, 200 µg mg^{-1}) were applied on the wounded area of each cotyledon. After 24 hour incubation at 27°C in darkness, eight representative cotyledons of each treatment were placed in

Millipore water (1 mL each). The water was spun down (10,000 rpm for 2 minutes) and the absorbance of the supernatant was measured at 285 nm (Shimadzu UV-1650PC). Test for normal distribution and Mann-Whitney U and accordingly Dunn's post-hoc test were performed using SPSS 17.0. To determine the relationship between absorbance at 285 nm and the actual concentration of mixed glyceollin isomers, decanted liquid samples from ten merged cotyledons were extracted with ethyl acetate treatment from the rest. After evaporation of ethyl acetate, samples were suspended in 100 µL methanol for HPLC analysis in an Agilent 1100 series device. Samples were analyzed by UV 285 nm detection through a 25 cm x 4 mm x 5 µm Lichrospher 100 RP-18 column in a gradient of methanol and H_2O (1mL min^{-1}). The gradient started from MeOH/H_2O (40%/60%), followed by a by a linear gradient to reach 100% MeOH at 20 min. A standard of purified glyceollin (0.1 mg mL^{-1}) was used for quantification. Furthermore, a liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) was performed with simultaneous DAD monitoring at 285 nm to investigate additional compounds that showed significantly different amounts between treatments. Samples were analyzed on a MaXis ESI-Q-TOF (Bruker Daltonics, Bremen, Germany) coupled with the same HPLC system as described before, in a gradient of MeOH and H_2O (containing 0.5% formic acid) using a flow rate of 700 µL min^{-1}. Full scan mass spectra (m/z 0-1000) were measured in positive ESI mode. LC/MS data was processed using Bruker Hystar software and analyzed with Bruker Compass Data Analysis (version 4.0).

5. Results

5.1. *In vitro* phenotypic variation of *B.gladioli* strain A

B. gladioli strain A colonies, isolated from female *L. villosa,* had smooth as well as rough morphologies (Figure 5).The smooth type colonies were beige, circular, shiny and raised with an entire edge. The rough type was whitish, undulated and wrinkled with serrated edges. The alignment of gyrase subunit B and 16S rRNA gene sequences gene showed a 100% sequence similarity, respectively. However, previous studies have shown that comparison of DNA gyrase subunit B gene (*gyrB*) sequence analysis provides higher resolution than 16S rRNA gene, to differentiate strain specificity (Tayeb et al., 2008; Wang et al., 2007; Yamamoto & Harayama, 1995). Since the metabolic gene make up is unknown, the original assortment of both phenotypes was used for experiments mentioning *B. gladioli* strain A. For comparison, the cultured *B. gladioli* strain G isolated from *L. hirta* also showed phenotypic variation (Flórez & Kaltenpoth, in preparation).

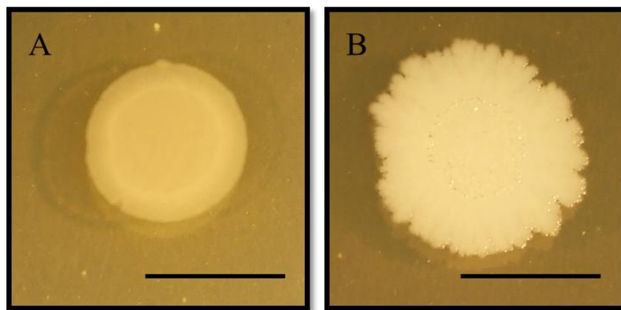

Figure 5. Different morphothypes of *Burkholderia gladioli* strain A recovered from a stock solution of dissected *L. villosa.* The pictures show the beige smooth type (**A**) and the whitish rough type (**B**) colonies 72 hours after incubation on KB medium. Scale bars represent 1 cm.

5.2 Localization of *Burkholderia* in the symbiotic organs of *L. villosa*

5.2.1. Adult females

Using fluorescent probes that target the 16S rRNA gene of eubacteria or specifically *Burkholderia*, we confirmed the localization of the symbionts within accessory glands associated to the reproductive system of the female *Lagria villosa,* as shown in Figure 6A. Although the presence of bacteria in these organs had been previously observed (Stammer, 1929), these had not been identified with molecular methods in

this species. *Burkholderia* was found in the structures in large amounts, as observed previously in the closely related host *L. hirta* (Flórez & Kaltenpoth, in preparation). The fluorescent signal from the eubacteria probe (EUB338-Cy5) colocalized to the *Burkholderia* specific probe (Figure 6 A), suggesting that all detected bacteria within the accessory glands belong to the genus *Burkholderia*, most likely to *B. gladioli*.

5.2.2. Larvae

In addition to diagnostic PCR screening, FISH was performed on cross-sections of an aposymbiotic larva, confirming the success of the symbiont removal method, as no bacteria were detected within the dorsal compartments (Figure 6 B). This larva was from the same clutch as those used for the horizontal acquisition experiment (chapter 5.3), (before exposure to *Burkholderia* infected leaves).

An additional larva that hatched from surface-sterilized eggs reinfected with the natural bacterial community (R-Nat) was also investigated for the presence of bacteria in the larval dorsal structures. Surprisingly, *Burkholderia* cells were not detected by FISH but other bacteria were found, as shown by the eubacterial probe labeling (Figure 6C). The shape of these bacteria was slightly different from *Burkholderia*, as visible under the microscope. These bacteria were smaller in size than typical *Burkholderia* rods and, morphologically, these were either cocci or short rods. Another R-Nat sample from the same clutch had *Burkholderia* as it was detected via diagnostic PCR (Table S). Solely two samples were available for comparison reasons, because all other individuals were reared until they reached adulthood.

Furthermore, FISH analysis of symbiotic larva with similar conditions resulted in a specific fluorescent staining with the *Burkholderia*, as well as the eubacterial probe (Figure 6 D).

B. gladioli strain A cultures were used as positive controls (data not shown). The specificity of the *Burkholderia* probe was tested previously with a range of other bacteria (Flórez et al., in preparation). Additionally, hybridization with a negative control probe (EUB3338_reversed-Cy5) resulted in no fluorescent signal in samples (data not shown).

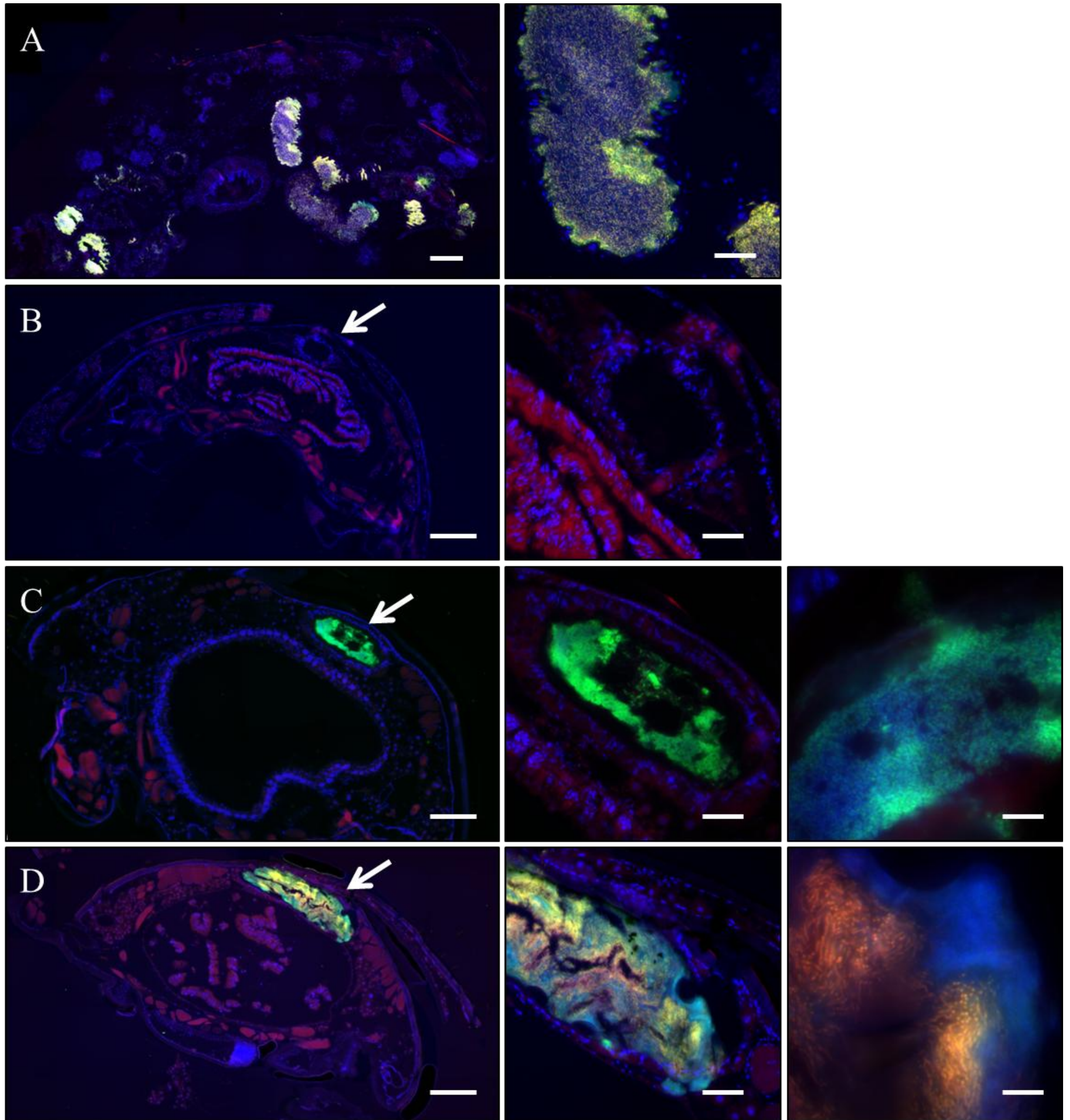

Figure 6. FISH on cross-sections of symbiont-bearing organs of *L. villosa*. (**A**) Symbionts are localized in accessory glands associated to the reproductive system of female imagines. (**B**) Dorsal compartments of aposymbiotic, (**C**) natural reinfected and (**D**) symbiotic larvae are shown (arrows). Magnifications of the symbiotic structures are placed on the right of each sample. All pictures show the merging of all channels, where *Burkholderia* specific labeling is shown in red (Burk_16S-Cy3), general eubacteria labeling in green (EUB338-Cy5), insect host nuclei labeling in blue (DAPI), and the yellow color represents the overlap between *Burkholderia* and eubacteria fluorescent signals. Scale bars represent 200 µm in the left panels, 50 µm in the middle panels and 10 µm in the right panels.

5.3 Horizontal transmission of the *Burkholderia* symbionts

5.3.1 Transmission of symbionts from *Lagria villosa* adult beetles to leaves

The occurrence of symbiotic *Burkholderia* was significantly different between leaf litter samples that had been in contact with *Lagria* beetles in comparison to controls as assessed by qPCR on the plant material (Student t-test, $p<0.0001$, Figure 7). Considering that five 16S rRNA gene copies are present per cell *in B. gladioli* strain A (Flórez et al., in preparation) there are on average 4.56×10^6 cells per mg of leaf litter. All of these cells were alive at the time of extraction as confirmed by cDNA generated by reverse transcription.

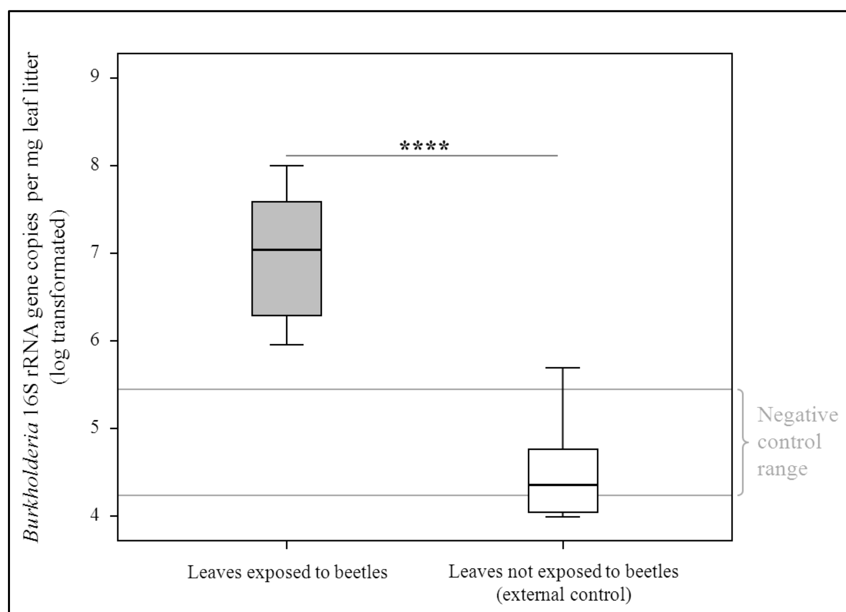

Figure 7. Horizontal transmission of symbiotic *Burkholderia* from *L. villosa* beetles to leaf material. Bacterial presence and abundance was assessed by quantitative PCR. Values of negative controls, obtained from the same qPCR run, are represented by gray lines. Asterisks indicate statistically significant differences ($p < 0.0001$, Student t-test).

5.3.2 Acquisition of *Burkholderia* from leaf litter by aposymbionts

Symbiont free *L. villosa* were exposed to *Burkholderia* between second and third larval instar until early adulthood. *B. gladioli* strain B, which is thought to be one of the naturally occurring (but uncultivated) symbiotic strains of *L. villosa*, was not detected in any sample. This was tested to assess whether the larvae were infected with a strain different to the one that was exposed to *L. villosa* within the experiment.

B. gladioli strain A was present in 85% of the individuals that were exposed to *Burkholderia* (Table 4). However, the analysis revealed also that 16% of the controls that were not exposed to *B. gladioli* strain A had *Burkholderia*.

Table 4. *Burkholderia* presence in different treatments of adult symbiotic organs of *L. villosa* females (from the same egg clutch).

Treatment	FISH	Diagnostic PCR	qPCR
Exposed to *Burkkholderia*	2/2	17/20*	17/20*
Not exposed to *Burkholderia*	0/2	2/12**	2/12**

* 7/17 positives with weak signals
** 2/2 positives with very weak signals

Additionally, *Burkholderia* cDNA quantification was performed to evaluate reinfection titers. The abundance of symbiotic *B. gladioli* was significantly different between individuals that were exposed to *Burkholderia* in comparison to controls (Mann-Whitney U test, $p < 0.0001$, Figure 8).

Although there was some amplification in no template controls (NTC), and of eight out of twelve control aposymbiont, the corresponding melting temperatures were on average 10°C less than the rest of the samples. Thus, the DNA concentrations of these samples were considered unspecific amplification and thus set to zero for *Burkholderia* quantification. Using *E. coli* as a negative control resulted in minor amplification at late cycles. Detected fluorescence signals of the samples that were weaker than these of *E. coli* controls were remarked as *L. villosa* individuals having no *Burkholderia* symbionts.

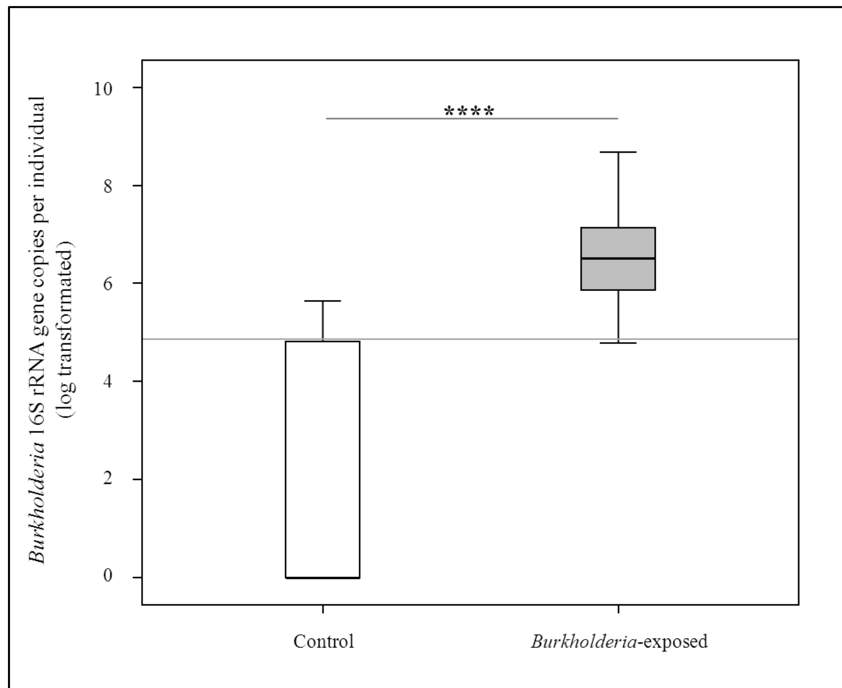

Figure 8. Abundance of *B. gladioli* strain A in a single accessory gland of female *Lagria villosa* after exposure to *Burkholderia*-infected leaves. The horizontal line represents *E. coli* DNA used as a negative control in the qPCR (gray line). Asterisks indicate statistically significant differences ($p < 0.0001$, Mann-Whitney U test).

Successful acquisition and localization of the symbionts inside the accessory glands of the female reproductive system was verified by FISH with *Burkholderia*-specific and general eubacterial probes. The bacterial cells in low to medium abundance (compared to Figure 6A) were present within the female´s accessory glands (Figure **9**A and B).

The aposymbiotic control with residual *Burkholderia* as assessed from qPCR (Table 4) and control aposymbiotic adults showed no fluorescent signals (Figure 9C and D). Higher autofluorescence of the Cy3 probe accounts for the red background as observed in Figure 9A-D.

For visualization purposes, only two *Burkholderia*-exposed individuals were screened by FISH. The first one, represented in Figure 9A and B, presents high abundance of *Burkholderia* as assessed by qPCR (4.84×10^8 16S rRNA gene copy no.), while the second one had a lower number of *Burkholderia* 16S rRNA gene copies (2.74×10^7). This second individual showed a few fluorescent spots, even though these were not clearly recognizable as bacteria.

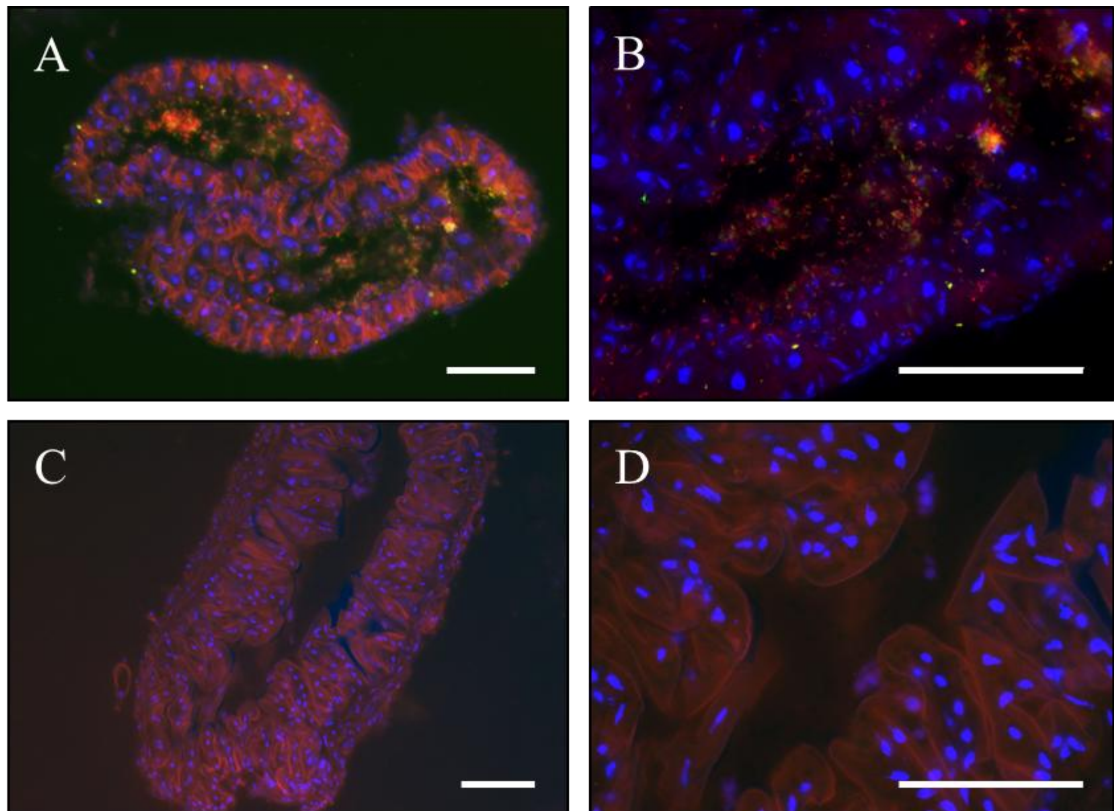

Figure 9. FISH on cross-sections (8 µm) showing the symbiont transmission organs of Lagria villosa. Accessory gland of (**A** and **B**) an individual exposed to Burkholderia and of (**C** and **D**) an aposymbiotic individual that was not exposed to Burkholderia. All pictures show the merging of all channels, where Burkholderia specific labelling is shown in red (Burk_16S-Cy3), general eubacteria labelling in green (EUB338-Cy5), insect host nuclei labelling in blue (DAPI) and yellow color represents the overlap between Burkholderia and eubacteria fluorescent signals. Single channel images of corresponding structures can be found in the supplementary (Figure S1). Scale bars represent 50 µm.

5.4 Interaction between *B. gladioli* and soybean plants

5.4.1 Systemic plant infection by *Burkholderia* symbiont of *L. villosa*

The presence of bacteria was assessed by PCR on soybean plants sampled 38 days posterior to inoculation. *Burkholderia* was detected in nearly all samples from inoculated plants, even in younger leaflets distant to the inoculation site (Figure 10). No *Burkholderia* were detected in untreated control plants. Difference areas of the leaves had different concentration of bacteria, which was quantified with qPCR using *Burkholderia gladioli* strain A specific 16S rRNA primers. The concentration of symbionts was highest around the site of infection, varying from 5.5 x 10^6 to 3.6 x 10^7 16S rRNA gene copies per mg of leaf tissue (five copies per cell). There

were lower bacterial concentrations found in the distant area on the same wounded leaf and in a distant younger leaf, as well. The amount of *Burkholderia* ranged from very low numbers of 2.2 x 10^3 to high numbers of 1.1 x 10^6 16S rRNA gene copies per mg leaf material. Amplification was also detected in low levels in untreated control leaves as well as in negative controls. This could be due to unspecific fluorescence signaling, e.g. based on primer dimer amplification, as often seen and described before (Dobosy et al., 2011; Mehndiratta et al., 2008; Nybo, 2011; Raso et al., 2011; Teo et al., 2002; Tichopad et al., 2010; Wen et al., 2009). However, there was a high significant difference between the treatments.

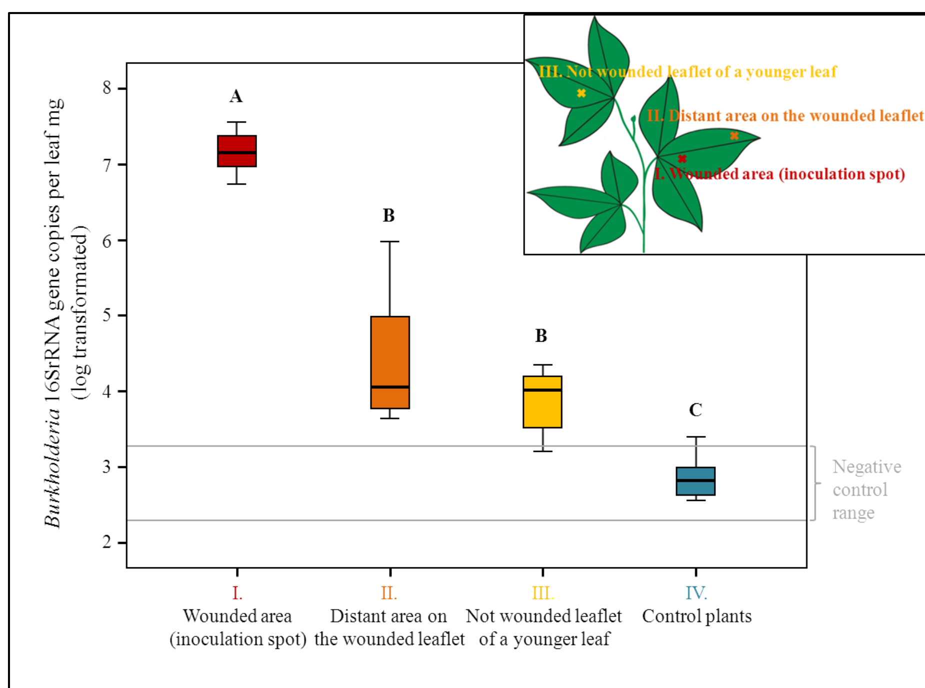

Figure 10. *Burkholderia* 16S rRNA gene copy numbers in different parts of treated soybean plants and controls. The schematic picture illustrates the sampling areas from *B. gladioli* inoculated plants (colored in red, orange and yellow). Samples from untreated control plants were taken from the inoculation spot (sterile tap H_2O). Values of negative controls, obtained from the same qPCR run, are represented by gray lines. The letters represent significantly different groups according to post-hoc tests (Tukey HSD, $p < 0.01$).

Since the presence of *Burkholderia* in different parts of the plant was established, we used FISH with a *B. gladioli* specific probe and a general eubacteria probe to visualize this systemic infection within the vascular system of leaf and stem. High abundance of bacteria was found in a 2 cm circular area of the inoculation site, whereas no bacteria were detected in a comparable spot of an untreated leaf

(Figur 11). The low bacterial fluorescence signals plus the higher autofluorescence of leaf tissue was mainly due to the thickness of the tissue. It seems like *Burkholderia* were not restricted to only phloem or xylem of the vascular system, rather they spread within the stem tissue (Figure 12). Additionally, *Burkholderia* were located in cross-sections on the surface of soybean leaves, close to the infection spot (Figure S2).

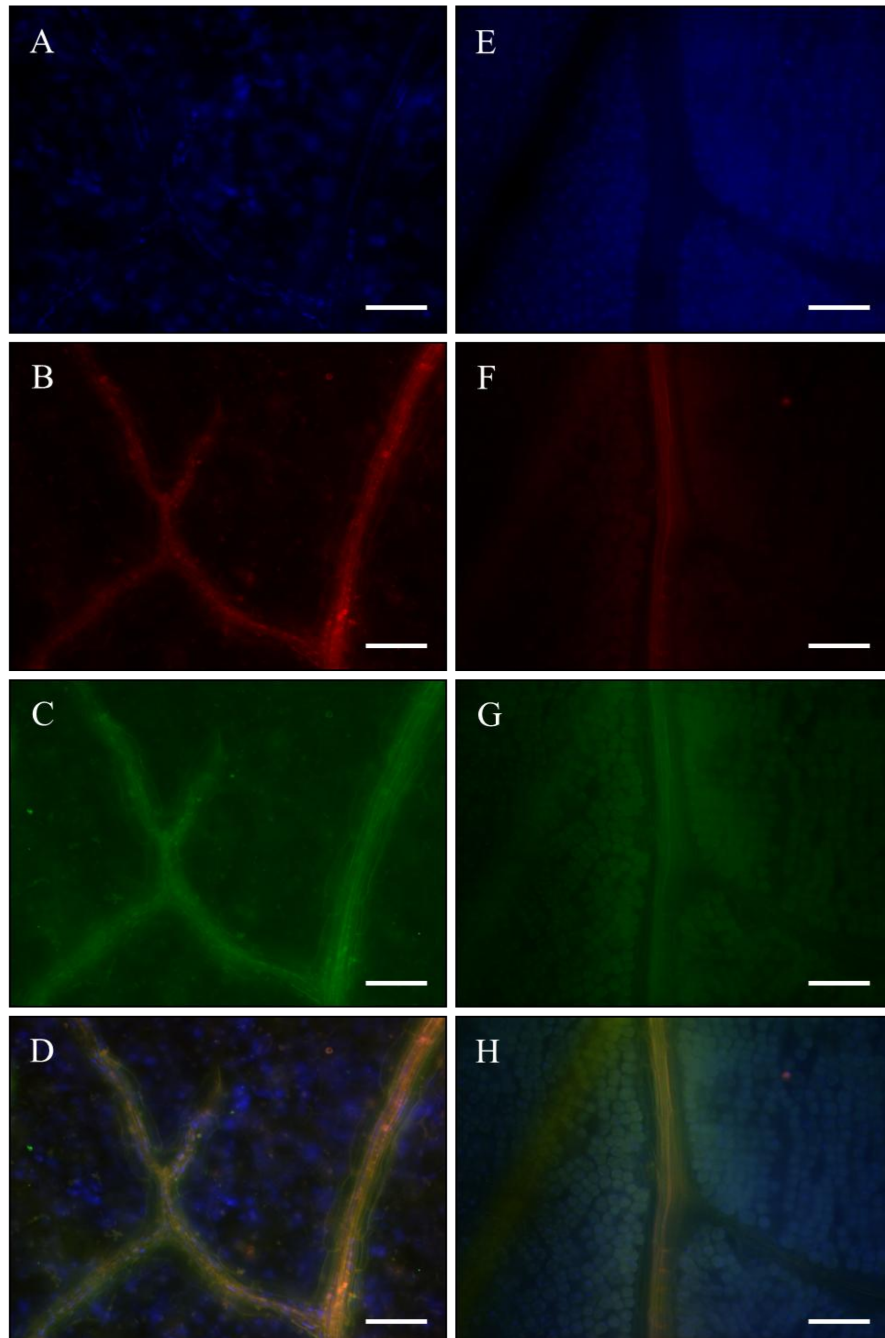

Figure 11. Whole mount FISH on soybean leaf tissues. **A** - **D** = *Burkholderia* inoculated plant. **E** - **H** = control plant**. A** & **E:** DAPI. **B** & **F**: Burk_16S-Cy3. **C** & **G**: EUB784-Cy5. **D** & **H**: Merge of all channels. Scale bars represent 50 µm.

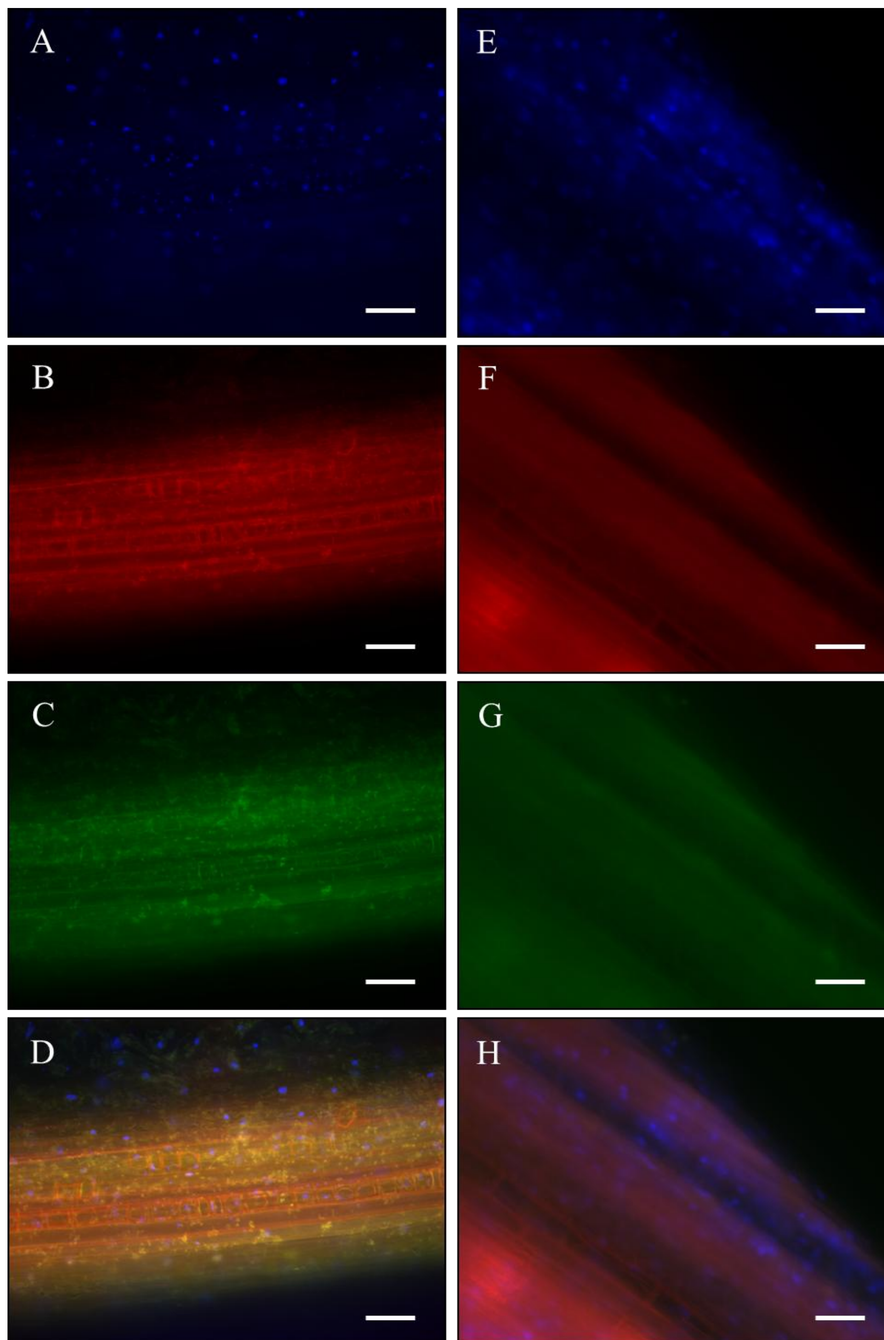

Figure 12. Whole mount FISH on soybean stem tissues. **A** - **D** = *Burkholderia* inoculated plant. **E** - **H** = control plant**. A & E:** DAPI. **B** & **F**: Burk_16S-Cy3. **C** & **G**: EUB784-Cy5. **D** & **H**: Merge of all channels. Scale bars represent 50 µm.

5.4.2 Impact on plant fitness upon infection with the symbiotic *B. gladioli*

Height, dry weight, seed production, chlorophyll content of the infected leaves, time of first flowering and leaf fall were used as proxies to assess the impact of infection by *Burkholderia* on the soybean plants. Reduced seed output was observed in

infected plants (Mann-Whitney U test, $p < 0.01$, Figure 13). In comparison to control plants there was around 17% less seed production in the infected plants. Moreover, *B. gladioli* infection showed significantly earlier flowering (Mann-Whitney U test, $p < 0.01$, Figure 14).

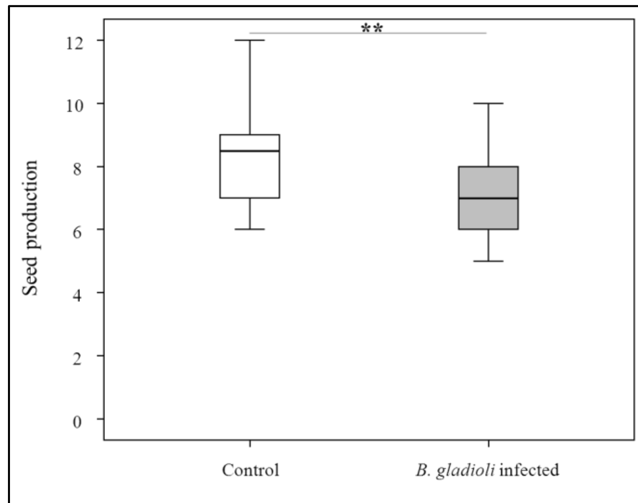

Figure 13. Seed output (count) of soybean plants treated with and without *B. gladioli* strain A. Asterisks indicate statistically significant differences (Mann-Whitney U test, $p < 0.01$).

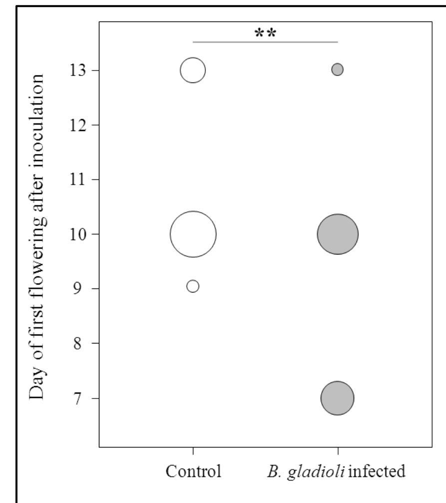

Figure 14. First flowering event of soybean plants after *B. gladioli* strain A inoculation in comparison to control plants. The radius is proportional to the no, of individuals that flowered at this time point firstly. Asterisks indicate statistically significant differences (Mann-Whitney U test, $p < 0.01$).

Significant differences between leaf chlorophyll content were observed between control and infected treated plants in the older leaves (GLMM, $p < 0.01$, Figure 15A), while younger leaves showed no significant differences (Figure 15B). There were no statistically significant differences ascertainable during the first ten days, but after this "initial phase", the chlorosis of older leaves was affected by bacterial infection. Nevertheless, there was neither hypersensitive response nor typical compatible plant-pathogen interaction in form of dramatic necrosis during the experiment. The bacterial invader caused no typical disease symptoms like blight or plant death.

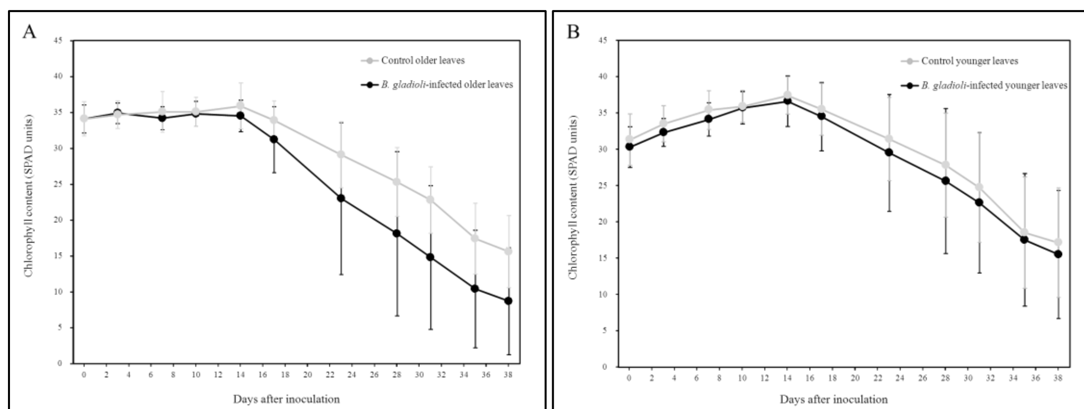

Figure 15. Relative chlorophyll values of (**A**) older leaves and (**B**) younger leaves of soybean plants *treated* with *Burkholderia (infected)* and sterile tap H_2O (control). Chlorophyll content was measured with a SPAD 502 Plus chlorophyll meter, where the numerical SPAD units are proportional to the absorbance at the corresponding wavelengths for chlorophyll. The means and standard deviations (error bars) of 18 replicates are shown.

Furthermore, there was a significant difference in the growth between plants with different treatments (GLMM, $P < 0.05$, Figure 16). The *B. gladioli* infected soybean plants grew about 16% higher than the non-infected control plants at the end point of the experiment. Biomass production (dry weight of plant tissue without roots) and the time of first leaf fall were not significantly affected by the bacterial infection (Figure S3 & S4).

In a second experiment using three different *B. gladioli* strains and *E. coli* K-12 (Table 3), visible necrosis or drastic chlorosis were not observed.

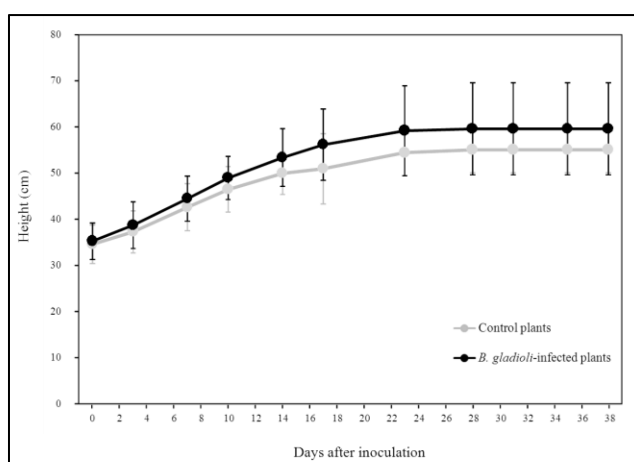

Figure 16. Height development of soybean plants treated with and without *B. gladioli.* The means and standard deviations (error bars) of 18 replicates are shown.

5.4.3 Phytoalexin response in soybean upon *Burkholderia* infection

A cotyledon assay was performed to investigate if soybean recognizes and responds to symbiotic *B. gladioli* as a pathogen. The phytoalexin response is observed as a red coloration appearing after exposure to a pathogen, which is correlated with the production of glyceollins by *G. max* as a defense mechanism. Glyceollins themselves are colorless compounds, but their precursors, including glycinol (THP) and glyceollidin I & II, have a red coloration (Ingham et al., 1981; Zahringer et al., 1981). We could observe a phytoalexin response upon treatment with two of the *B. gladioli* strains qualitatively (Figure 17) and quantitatively (Figure 18A & B).

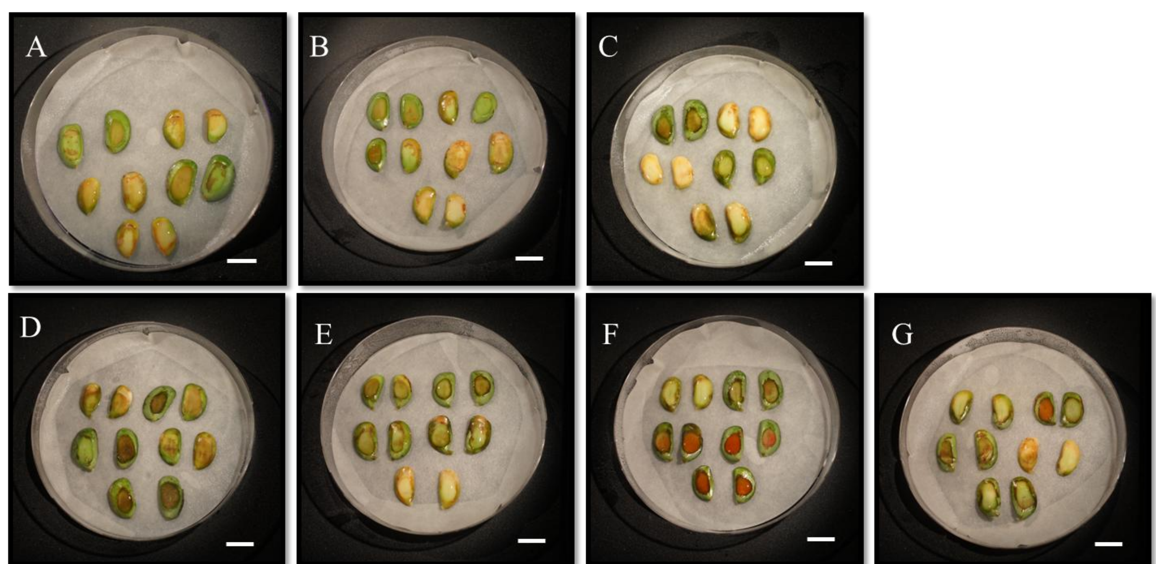

Figure 17. Cotyledon assay for the detection of phytoalexin production, particularly the *G. max*-specific glyceollins. Seven different treatments consisting of either bacterial cell suspensions or controls are shown. (**A**) H_2O, (**B**) *E. coli* and (**C**) inactivated *B. gladioli* strain A were used as negative controls, and (**D**) ß-glucan isolated from *P. megasperma*, known to elicit phytoalexin reaction, was used as a positive control. Bacterial solutions containing 10^6 CFUs of (**E**) *B. gladioli* strain G isolated from *L. hirta,* (**F**) *B. gladioli* strain A isolated from *L. villosa,* and (**G**) human pathogenic *B. gladioli* pv. *cocovenenans,* respectively, correspond to the infection treatments. Scale bars represent 1 cm.

In addition to direct visual assessment of the red coloration, glyceollins were extracted and quantified based on absorbance at 285 nm. The highest absorbance was monitored for the symbiotic *B. gladioli* strain A isolated from *Lagria villosa* (Figure 18). Surprisingly, there was no significant reaction observed for the other symbiotic *B. gladioli* strain G which is originally isolated from the European species *Lagria hirta*. Host plant specificity may be responsible for lower pathogenicity.

However, *B. gladioli* pv. *cocovenenans* known as a human pathogen (Coenye et al., 1999) caused a phytoalexin response. The absorbance is not significantly different to the reaction caused by symbiotic *B. gladioli* strain A. A similar pattern was revealed in the elicitor treatment. The negative control treatments, including H_2O, *E. coli* and dead *B. gladioli* strain A, caused significant reaction. Only in some of these cotyledons slight darkening was visible, which is explained as normal stress response due to wounding or dryness (Darvill & Albersheim, 1984).

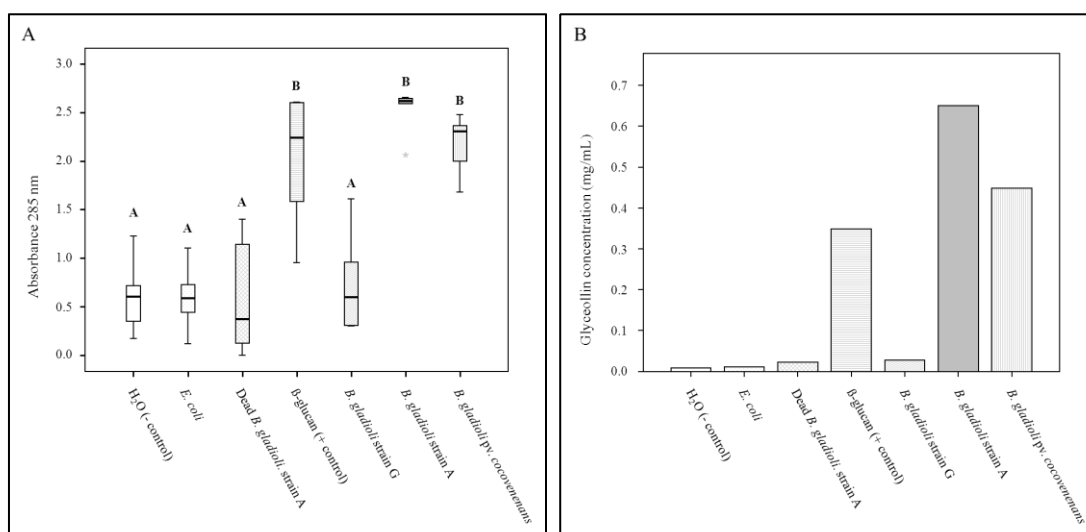

Figure 18. Phytoalexin quantification of soybean cotyledons induced by different bacterial inoculations or elicitors, respectively. (**A**) Concentration of phytoalexins is expressed as absorbance at the corresponding wavelength for this compound, 285 nm. Letters indicate statistically significant grouping (Mann-Whitney U test, $p < 0.01$ and Dunn post-hoc test, $p < 0.05$). (**B**) Corresponding glyceollin concentrations extracted from the wound-droplets of ten merged soybean cotyledons per treatment. Concentrations were calculated based on a known standard.

To confirm the previous results, the experiment was repeated under the same conditions and ten representative cotyledons from each treatment were extracted and ran in a high-performance liquid chromatography (HPLC) equipped with a UV detector. Each treatment showed two peaks corresponding to a mixture of five different glyceollin isomers which were quantified using a glyceollin standard isomer mixture (Figure 18 & S7). The glyceollin standard was run as an additional sample. The direct quantification results support the results obtained previously by direct UV-Vis absorbance. Low glyceollin concentrations in the control treatments are possibly caused by wounding of the cotyledons, since normal stress responses can account for background production of the compounds.

The THP concentration, a direct precursor of glyceollin, was much higher in the *B. gladioli* strain A and *B. gladioli* pv. *cocovenenans* treatments (Figure S5 & S7). Unfortunately, there was no THP standard concentration available. Furthermore, two unknown compounds were produced in strikingly higher amounts in cotyledons treated with *B. gladioli* strain A and *B. gladioli* pv. *cocovenenans*. LC-MS analysis revealed peaks at about 10.5 min and 12.4 min with a molecular ion at *m/z* 271.06 and 255.07, respectively (Figure S6). These compounds are likely other precursors present in the glyceollin biosynthesis pathway or antimicrobial isoflavones. Candidates for the first substance are 2'-hydroxydaidzein and genistein, a trihydroxylated isoflavone that accumulates in soybean tissues during incompatible plant-pathogen interactions (Carpentieri-Pipolo et al., 2005; Fett & Jones, 1984). The second substance is potentially daidzein, a dihydroxylated isoflavone and glyceollins precursor.

6. Discussion

6.1 Phenotypic variation of symbiotic *B. gladioli* strain A

Two different colony phenotypes were observed in the *B. gladioli* isolated strain A, a smooth-yellowish and a wrinkled-whitish type (Figure 5). Although the partial sequences of two marker genes (16S rRNA and *gyrB*) are identical, we cannot be completely sure that the two phenotypes are genotypically identical. However, it was seen in the cultured *B. gladioli* strain isolated from *L. hirta* that the two different phenotypes represent also most likely the same genotype (Flórez, unpublished). The smooth morphotypes grew slightly slower and became a bit more wrinkled in old colonies as it was previously reported for *B. gladioli* by Stoyanova (2011). Nevertheless, both phenotypes remained different in matured cultures. They differed in color, opacity, shape, and surface properties. That leads to the assumption that both types may have different biochemical characteristics, which could be important for the expression of bacterial traits, e. g. pathogenicity, persistence or survival (Sousa et al., 2011). Fiori et al. (2011) also reported two different phenotypes of *B. gladioli* pv. *gladioli* isolated from saffron. They found a smooth, white-cream colony type and a wrinkled, green-yellow colony type, which show differences in metabolism, utilization of carbon sources, as well as in pH- and salinity-tolerance. The wrinkled phenotype may have advantages over the smooth type through survival in acidic and saline environments. In *P. fluorescens* it was documented that wrinkled colonies have fitness advantages through the production of cellulose-based biofilms and the expression of factors that could be important for attachment to surfaces as well as for persistence in dehydrated conditions (Spiers, 2007, 2014). Furthermore, smooth morphotypes of *P. aeruginosa* were reported to have lipopolysaccharides (LPS) and surface proteins, whereas their wrinkled counterparts did not (Mortensen et al., 2009). LPS, especially the lipid A (endotoxin) and the O-antigen, are known as virulence factors and may play major roles in symbiotic associations. Interestingly, symbiotic *Burkholderia* of the bean bug *R.pedestris* express specific virulence factors when cultured, but not in the insect gut. The host-associated symbionts lack the LPS O-antigen and were more susceptible to antimicrobial peptides that were produced by the host (Jiyeun Kate Kim et al., 2015). Thus, the interaction between *R. pedestris* and their environmentally acquired *Burkholderia sp.* could induce changes in the

bacterial cell membrane allowing the establishment of the symbiosis. In a more general context, it is possible that adaptation to variable environmental conditions is supported by phenotype heterogeneity in bacterial populations (Avery, 2006; Smits et al., 2006), and that phenotypic switching could play a role for potential symbionts, particularly *Burkholderia* (Sousa et al., 2011).

6.2 Horizontal transmission of *Burkholderia* symbionts

6.2.1 Transmission of symbionts from *L. villosa* adult beetles to leaves

I found out that lagriid beetles transmit their bacterial symbionts to leaf litter and that these can remain and survive on the leaf material (Figure 7), which is essential for future uptake by new hosts. The results from this study are in line with earlier findings which demonstrated that soybean leaves of living plants that had been in contact with female *Lagria* beetles contained *Burkholderia*, while control leaves that were not exposed to beetles showed none (Flórez et al., in preparation).

Bringing both results together leads to the conclusion that plants could be a source or an intermediate reservoir of symbiont transmission to new hosts. *B. gladioli* was previously reported as an endophyte in soybean (Kuklinsky-Sobral et al., 2005), thus it is likely that they can maintain an endophytic lifestyle and persist inside intercellular spaces or the vascular system as it is common for endophytes (Hallmann et al., 1997; Hardoim et al., 2015). Within this study, the symbiotic *B. gladioli* infected the soybean plant systemically (Figure 10) and were detected by FISH visualization within the vascular system (Figure 11 & 12). These findings suggest that the symbiotic *Burkholderia* rather consume metabolites produced by the host plant than degrade plant material. However, symbiotic *B. gladioli* are possibly able to synthesize plant material degrading enzymes as has been reported for other *B. gladioli* strains (Seo et al., 2015). This could be especially relevant for living in or on dead plant material that does not produce any metabolitic nutrients, which can be consumed by bacteria. Furthermore, there is evidence that symbiotic *B. gladioli* can persist on the leaf surface (Figure S2), which is likely supported by biofilm formation and utilization of degraded plant carbon and nitrogen sources (Gnanamanickam & Immanuel, 2006).

6.2.2 Acquisition of symbionts and their translocation to the symbiont housing organs

The localization of the *Burkholderia* symbionts of *L. villosa* was confirmed by performing FISH on the accessory glands of the female reproductive system as well as within the larval dorsal compartments (Figure 6A & D). These results support the dominance of *Burkholderia gladioli* that was previously observed using high throughput pyrosequencing and qPCR data (Flórez & Kaltenpoth, in preparation).

Furthermore, it was revealed that the natural reinfection (R-Nat) of eggs does not always favor predicted symbiotic associations. Instead of the most abundant *Burkholderia* symbionts, other unidentified bacteria were observed within the larval symbiotic organs in the individual that were used for FISH (Figure 6C). However, *Burkholderia* DNA was detected by diagnostic PCR in the second individual (Table S). One explanation could be that not all eggs were reinfected consistently with the natural bacteria consortium. Alternatively, it is possible that Burkholderia had lower chances of surviving in the PBS wash used for reinfection, or that the physical conditions on the egg changed after sterilization and differentially affect reinfection by the bacteria. The reasons still remain unclear and more extensive research on this should be carried out in the future, also evaluating which other bacteria were in the egg wash or could be present in the adult female R-Nat individuals, e.g. by using other techniques like molecular cloning or pyrosequencing.

Aposymbiotic larvae were generated and used for the test of potential *B. gladioli* acquisition from the environment. The method of generating symbiont free individuals was successful in this study. The aposymbiotic individual harbored no bacteria within the dorsal compartments of the larva as has been shown by FISH (Figure 6B). In addition, there were no *Burkholderia* found by diagnostic PCR in six aposymbiotic larvae, supporting these results (Table S). Same results were obtained in accessory glands of two adults, using fluorescence in situ hybridization. However, *Burkholderia* were detected in symbiotic accessory glands of one of five aposymbiotic adult individuals by using diagnostic PCR, suggesting that not all individuals were completely symbiont-free or they probably acquired *Burkholderia* from the environment during laboratory rearing (Table S).

The results of the horizontal transmission experiment suggest a high rate of symbiont acquisition from the environment. About 85% of tested female aposymbionts of *Lagria villosa* that were exposed to *Burkholderia* until they reached adulthood carried symbionts within the accessory glands of the reproductive system. Although a few *Burkholderia* cells were observed in the accessory glands of aposymbiotic beetles that were not exposed to *Burkholderia* (Figure 8), this was the case for two out of twelve evaluated individuals and the bacterial abundance was overall significantly lower than in naturally infected beetles. This could mean that the sterilization procedure does not eliminate 100% of the bacteria, or that the bacteria were taken up from the environment despite the controlled conditions. However, when compared to the mean of the individuals that were exposed to *B. gladioli*, there was about 26 times less abundance of *Burkholderia* 16S rRNA copies in the controls. Likely, the symbionts are acquired during the larval stage and maintained as well as integrated to the transmission organs during the pupal stage. Not much is known about the persistence of symbionts in coleopteran hosts during metamorphosis. For gut-associated microbes, it is assumed that only a minority of symbionts are maintained during the pupal stage like it was shown in bark beetles (*Ips Pini)*, in which the gut symbionts were maintained across all life stages with pronounced declines during the pupal stage (Delalibera et al., 2007). However, the route of horizontal transmitted *Burkholderia* symbionts to the accessory glands of the female reproductive system from larval to adult stage remains elusive. There is still nothing known about the fate of *Lagria* symbionts during metamorphosis from the larval dorsal compartments to the adult accessory glands even for those coming from the egg surface during vertical transmission (Buchner, 1965; Stammer, 1929). Although the dorsal compartments of the larvae are not connected to the gut, they are very closely located to it. Maybe some symbionts migrate into the gut lumen shortly before or shortly after reaching the pupation phase and passed a posteriori to the newly constructed accessory glands. In fact, the presence of several *Burkholderia* cells was detected in newly constructed imaginal gut epithelium of male *L. hirta* pupa by FISH (Flórez & Kaltenpoth, in preparation). One hypothesis for an environmental acquisition route is that *Burkholderia* are acquired orally from the leaf diet and maintained inside the gut. Therefore, they have to find a way to migrate actively from the opening of the anus into to the symbiotic organs, because a direct migration through the gut epithelium into the symbiotic accessory glands seems to be

nearly impossible (Buchner, 1965). Hypothetically, there must be a specialized control mechanism of the females to maintain the proper symbionts. In the model lepidopteran *Galleria mellonella* the gut microbiota is controlled during metamorphosis by the host immune system by the production of lysozyme, which interact synergistically with antimicrobial peptides produced by the symbiont (Johnston & Rolff, 2015). Furthermore, the expression of compounds like coleoptericin A (Login et al., 2011) and different pattern recognition receptors (Ratzka et al., 2013) by the host, play key roles during pupal phase in controlling and maintaining symbiont associations. In addition, the expression of other defensive proteins (Futahashi et al., 2013; J. K. Kim & Lee, 2015; Ohbayashi et al., 2015) and the bacterial cell motility seem to be basic requirements in the establishment of symbiosis by *Burkholderia* in hemipterans (J. B. Lee et al., 2015). It may also be possible that some residual bacterial cells from the gut microbiota that are not shed with the old gut are released to the hemocoel during metamorphosis and then bacterial cells migrate actively to the accessory glands. This could be triggered by chemotactical compounds produced by the host, although this is purely speculative.

Buchner (1965) speculated on a possible *Lagria* symbiont transmission that is likely similar to that of *Haematopinus* during the last molting step, which also harbors symbionts in three dorsal compartments of the embryo, however, of hypodermal origin. The symbionts are released from the dorsal compartments to the outside into a liquid-filled space that is located between the old and the new cuticula and then subsequently pass from it through the molting fluid to a wide opening of the sexual rudiment and then through the latter to the ovarial ampullae.

Hence, the most likely hypothesis is that *L. villosa* larvae acquire *Burkholderia* from the environment during the process of molting enforced by active migration into the dorsal compartments and not by feeding on *Burkholderia* infected leaves. The ablity of *B. gladioli* to secrete chitinase could thus play a role (Chowdhury & Heinemann, 2006; Kong et al., 2001; Shimosaka et al., 2001), since the dorsal compartments as well as the cuticle are lined with chitin. Both structures, the three dorsal compartments of the larvae and the imaginal accessory glands, resulted from invaginations of the embryonic and pupal cuticle, respectively (Stammer, 1929). During the larval molting process the cuticle firstly opens on the spots where the dorsal compartments are located. On account of this, it´s likely that *Burkholderia* could have remained on the cuticle and subsequently migrated into the symbiotic

structures easily. Similar to the transmission route in *Haematopinus,* the symbionts could have been passed through the molting fluid to the intersegmental openings of the old cuticle, which later become the symbiotic accessory glands of the reproductive system, based on descriptions in *L. hirta* (Stammer, 1929; Tschinkel & Doyen, 1980).

Furthermore, it is possible that horizontal transmission events occur under natural environmental conditions during the egg stage of *L. villosa*. *Burkholderia* uncovered eggs could acquire symbionts from leaf or soil material and the symbionts could migrate into the larvae as described by Stammer (1929). As acquisition of microbial symbionts from the egg surface by larvae or nymphs has been recognized as part of a common vertical transmission route in insects (Bright & Bulgheresi, 2010; Salem et al., 2015), it is possibly also an opportunity for horizontal acquisition. Symbiont acquisition in later developmental stages of insects has been demonstrated only in a few systems. *Burkholderia* symbiont establishment happened most successfully in *R. pedestris* nymphs during the second instar when mid gut crypts are completely developed (Kikuchi et al., 2011b). Another exception is reported in termite-symbiont associations, wherein gut symbiotic bacteria and protists are acquired after every molting step via feeding of anal excrements from colony mates (Inoue et al., 2000).

6.3 Soybean plant-*Burkholderia* interaction

6.3.1 Effect of *Burkholderia* on soybean plants

Though *B. gladioli* have been known as pathogens in several plant species, a pathogenic impact on soybean could not be reported previously.

The results provided evidence for a systemic infection of the insect symbiotic *B. gladioli* strain A in soybean plants. *Burkholderia* presence was detected in stem tissues and in leaf tissues on the infection sites as well in distant leaflets within the vascular system (Figure 10 - 12). The highest abundance of *B. gladioli* (in mean 2.9 x 10^9 cells g^{-1} fresh weight) was found on the inoculation spot (Figure 10), and the bacteria also persisted on the leaf surface (Figure S2). In a distant region on the inoculated leaflet, *Burkholderia* presence was about 100 times less. Similar results were seen in distant younger leaves, which were not inoculated with *Burkholderia* (in mean 1.5 x 10^6 cells g^{-1} fresh weight). It might be that *B. gladioli* grow preferably in specific parts of the plant and disperse through the vascular system. Therefore, this

could increase the chances that the symbionts are able to affect the plant physiology in different organs or in general. This study focuses on the leaves, because *Lagria villosa* beetles mainly feed and reside on the leaves and thus could acquire symbionts from the plant. Whether they could be localized in root, seed or other plant tissues has therefore not been tested.

For comparison, common population densities of plant pathogenic bacteria were reported above threshold levels ranging from 10^7 cells g^{-1} fresh weight, e.g., for *Clavibacter michiganensis* subsp. *sepedonicus* on tomato (Tsiantos & Stevens, 1986), to 10^9 - 10^{10} cells g^{-1} fresh weight, as reported for *Pseudomonas solanacearum* on tomato, eggplant and pepper (Grimault & Prior, 1994). In contrast, general abundances of harmless endophytic bacteria were found far below these ranges between 10^3 to 10^5 cells g^{-1} fresh weight, e.g. for *Pantoea agglomerans* on sugarcane (Quecine et al., 2012) and for *Eneterobacter asburiae* on cotton and bean (Quadt-Hallmann & Kloepper, 1996). Higher population densities of bacterial endophytes above 10^9-10^{10} cells g^{-1} fresh weight are also reported, but exclusively for complex bacterial communities, e.g. on sweet corn and cotton (McInroy & Kloepper, 1994). However, phytopathogenic bacteria of *Xylella fastidiosa* causing no disease symptoms were also detected in lower densities, ranging from10^5 - 10^6 cells g^{-1} fresh weight at distant points to the inoculation spots on grapevine plants (Gambetta et al., 2007) and on mugwort and watergrass (Hill & Purcell, 1995) In conclusion, bacterial counts are not necessarily good indicators for pathogenicity, but can give hints about the impact of plant associated bacteria.

The systemic *Burkholderia* infection caused neither visible drastic disease symptoms nor a hypersensitive response (necrosis), indicating that under given conditions they were not highly virulent to the plant. Surprisingly, the high inoculum rate of *B. gladioli* strain A had no directly visible pathogenic impact. On account, it could be that the symbiotic *B. gladioli* has been rather recognized as an endophyte than as a plant pathogen. There are multiple definitions for bacterial endophytes. However, the most commonly used, defines endophytes as bacteria colonizing inner host tissues, without causing damages or eliciting strong defense responses (Hallmann et al., 1997). They can be found locally or systematically in plant tissues, sometimes in high numbers, but not inside living cells like endosymbionts. Historically, they have been thought to be weakly virulent or latent plant pathogenic (Hallmann et al., 1997).

However, increasing researches demonstrated recently the beneficial impact of specific bacterial endophytes promoting plant growth (Oteino et al., 2015; Subramanian et al., 2015; Touceda-Gonzalez et al., 2015; Xia et al., 2015) or reducing disease symptoms caused by plant pathogens (Gomez-Lama Cabanas et al., 2014; Gond et al., 2015; Mousa et al., 2015; Muzammil et al., 2014).

Although no visible disease symptoms were seen, the *B. gladioli* strain A infection caused decreased host fitness, expressed as lower seed production in soybean plants (Figure 13). Furthermore, infected plants started flowering significantly earlier (Figure 14), suffered from more pronounced chlorosis in older leaves (Figure 15) and grew higher than control plants (Figure 16). The accelerated flowering, chlorosis and growth could be due to the expression of plant growth promoting substances that were triggered by bacterial presence or secreted directly by the *Burkholderia*. Plant growth regulators such as auxins, cytokinins, ethylene, gibberellins and jasmonic or salicylic acid have been considered as causal agents for alterations in plant growth and development (Hallmann et al., 1997). Plant growth promotion has been more frequently considered in associations with rhizobacteria, which are also involved in plant beneficial nutrient uptake (Glick, 1995). There are also many examples of leaf endophytic bacteria producing plant growth promoting hormones (Bernabeu et al., 2015; El-shakh et al., 2015; Kurepin et al., 2015; Passari et al., 2015; Ryan et al., 2008). In addition, plant growth promotion could also be due to the suppression of deleterious microflora by the introduced endophyte (Zablotowicz et al., 1991). Some *Burkholderia* species are known to produce antimicrobial compounds that could be potentially beneficial for the host plant in symbiotic partnerships, mainly against pathogenic fungi (Attafuah & Bradbury, 1989; Elshafie et al., 2012). Another example, the *Psychotria* leaf symbiont *Burkholderia kirkii* produces metabolites that are thought to protect its host plant *P. kirkii* against pathogens or herbivores (Carlier & Eberl, 2012). Although accelerated development can be associated with positive effects on plant physiology, it has also been reported as a stress response upon pathogen infection of *P. syringae*, *P. parasitica* and *X. campestris* in the model plant *Arabidopsis thaliana* (Korves & Bergelson, 2003). In response to compatible pathogen infection, susceptible plants can exhibit increased levels of jasmonic acid, auxin, ethylene and salicylic acid (Dong, 1998; Lund et al., 1998; O'Donnell et al., 2003).

Importantly, the *B. gladioli* infection resulted in lower seed output by the soybean plants, suggesting that bacterial invasion was costly for the host plant. This could be due to different reasons, e.g. the bacteria deprive nutrients of the plant or the plant invests energy in a defense response at the expense of reproduction. Considering that the bacteria infected different parts of the plant, the faster rate of chlorosis could be a manifestation of limited resources as well, but also a direct consequence of pathogenesis. There is a chance that the symbiotic *B. gladioli* cause disease under physiological stress, like limited nutrient conditions, as resource availability can trigger virulence (Snoeijers et al., 2000). As far as it is known, endophytic bacteria have not been reported to cause fitness deficits in symbiotic associations with their host plants (Friesen et al., 2011). However, it has been assumed that endophytic bacteria could evolve to harmful organisms, especially when the symbionts are transmitted horizontally (Sachs et al., 2010). Therefore, this study prefers to consider the symbiotic *B. gladioli* as a plant pathogen with low virulence.

6.3.2 Defensive reaction of the host plant

Glyceollin or isoflavon production was only detected on the wounded soybean cotyledons inoculated with the *L. villosa* symbiotic *B. gladioli* strain A and with the pathovaric strain *cocovenenans* (Figure 17 - 18 & S5 & S7).This last one is known as human pathogenic, but not described as a plant pathogen (Coenye et al., 1999). However, the pathovar *cocovenenans* produces toxins that can, theoretically, act as phytopathogenic factors. Bonkrekic acid and toxoflavin are also known as virulence factors against plant mitochondria (Iiyama et al., 1998; Passam, 1975). The glyceollin response reveals that the symbionts were recognized by the early-stage plant. It is known that glyceollins are produced also in leaves as consequences of incompatible as well of compatible pathogen-host interactions (Keen, 1978). However, it is possible that the host plant is more tolerant to the bacterial pathogenic traits at later life stages. Thus, further experiments are necessary to conclusively determine if there is a defense response in older plants to pathogenic invasion of the symbiotic *B. gladioli*.

Surprisingly, no glyceollins were detected in the cotyledons inoculated with the *Lagria hirta* symbiotic *B. gladioli* strain G. This could be due to a different recognition by the plant receptors. Furthermore, it is possible that the *B. gladioli*

strain G is not able to produce plant pathogenic metabolites. Flagellin and surface molecules like LPS or EPS are most often the elicitors of pathogen recognition (Sequeira, 1985; Zipfel, 2014). However, in a previous study was found out that LPS and EPS, isolated from two different plant pathogenic *B .gladioli* strains, caused no visible reactions on tobacco leaf tissues, but extracted toxoflavin, isolated from the same strains, caused necrotic lesions (Furuya et al., 1997b). Supporting these findings, no glyceollin production was considered in the cotyledons inoculated with dead *B. gladioli* strain A, leading to the assumption that surface molecules are not crucial as inducers of the higher glyceollin production as seen in the assay with live cells. Hence there must be a pathogenic elicitor produced by living bacterial cells, like a toxin (Vidhyasekaran, 2008). Toxoflavin is assumed to be a factor with high plant pathogenic impact (Angus et al., 2014; Furuya et al., 1997a; Seo et al., 2015). This compound is known to cause chlorotic damage to panicles of rice plants and inhibits the growth of rice's leaves (Jeong et al., 2003; Suzuki et al., 1998; Yoneyama et al., 1998). In a previous study, it was clearly demonstrated that almost all toxoflavin producing strains of *B. glumae* were virulent to rice plants, whereas all toxoflavin non-producing strains were avirulent (Iiyama et al., 1995). Furthermore, in *B. glumae* was shown that toxoflavin synthesis does not start until cells reach high densities (J. Kim et al., 2004) or in dense inoculates (Chen et al., 2012). Furthermore, there are indications that toxoflavin production is under control of the quorum sensing (QS) system (J. Lee et al., 2016). Possibly, absence or regulation of QS genes in the *Lagria hirta* symbiont (*B. gladioli* strain G) result in deficient toxoflavin production. Additionally, there were also no disease symptoms in the soybean plants that were infected with the *B.gladioli* strain A isolated from *Lagria villosa*. Consequently, it could be likely that there was no toxoflavin production in the soybean plants because of a downregulation of the QS genes, caused by less cell density or single cell separation.

In addition, it would be interesting to identify the two unknown remaining compounds that were produced by the cotyledons as a reaction to the presence of the *B. gladioli* strain A and pv. *cocovenenans*. These compounds could represent potential antibacterial defensive responses of the soybean plant. They are probably precursors of the glyceollin synthesis pathway, probably daidzein or other isoflavonoids likely genistein.

It would also be interesting to test in future studies whether the symbiotic *B. gladioli* strains induce phytopathogenic symptoms on other plant species. In addition, it should be noted that the results in this thesis show the pathogenic potential of one single strain of the *Lagria-Burkholderia* symbiosis that was isolated from *L. villosa* and *L. hirta*, respectively. There are other still uncultivated mutualistic *B. gladioli* strains of *L .villosa* and *L. hirta* (Flórez & Kaltenpoth, in preparation) that could have different pathogenic traits and effects on host plant fitness. In further studies these other types should be investigated.

6.4 Tripartite interaction between the insect, the plant and the bacteria

Burkholderia gladioli can be found in various environmental niches. They have adapted to different conditions and are able to switch between plant- and insect-associated as well as free-living lifestyles. *Lagria* beetles are able to transmit their symbionts to both soybean plants and dry leaves of pea, raps and soybean plants, where they can persist. It is possible that the leaf wounding or also degrading of leaf material facilitates a higher bacterial colonization within the host plant as it was reported in other systems (Humphrey et al., 2014). Therefore, the systemic colonization of the soybean plants seems to be likely a route for horizontal transfer of insect mutualistic *Burkholderia* to new lagriid hosts. Given their plant pathogenic ancestry (Flórez et al., in preparation), it is comceivable that the *B. gladioli* symbionts of the lagriids often also live associated to plants either as endophytes or pathogens. Considering that the bacteria were not recognized by adult soybean plants as pathogenic, it seems as if they have lost their full plant pathogenic potential. They were neither killed through hypersensitive response that would be expressed as an incompatible reaction, nor did they induce a compatible reaction that would result in the death of the host plant. Nevertheless, they could still remain as latent phytopathogens and become more virulent under specific environmental conditions.
Several phytopathogenic bacteria are transmitted via insect vectors (Nadarasah & Stavrinides, 2011; Weintraub & Beanland, 2006). Also, symbionts of some insect herbivores can manipulate host plant physiology and can alter anti-herbivory defenses in plants, facilitating food resource usage by the insect (Frago et al., 2012). In this study, the systemic infection of the *L. villosa* symbiotic *Burkholderia* affected

plant reproductive fitness and development. There was significantly earlier flowering, increased chlorosis in older leaves and enhanced plant growth. Overall, this probably leads to earlier leaf fall and thus increased availability of larval leaf diet. However, this hypothesis is largely speculative and if present at all, only a side effect, as it is unlikely that such a strategy evolves given the high chance of cheaters to exploit resource availability.

It is also likely that *B. gladioli* produces antimicrobial compounds that are beneficial not only for the insect hosts by antifungal egg protection (Flórez et al., in preparation), but also for the plant hosts. Although, it is costly for the plant to have a non-very harmful symbiont, it could be advantageous for the host to harbor an agent with antifungal activity in the presence of phytopathogenic fungi. There are some *B. gladioli* strains that have the potential to protect plants against fungal parasites, although this has only been demonstrated *in vitro* (Elshafie et al., 2012; Jha et al., 2015; Yan et al., 2015). Therefore, it might be beneficial for the host to receive protection from the *Burkholderia gladioli* symbiont when exposed to naturally occurring fungi, given that the bacteria causes neither drastic disease symptoms nor outweighing fitness deficits.

For this reason, it should be examined in future whether they could also play a role in defense of host plants against bacterial or fungal antagonists. That would be also beneficial for *Lagria villosa,* which live in close contact to plant material from the egg to the imago stage, since feeding on leaves containing detrimental bacteria or fungi might be unfavorable for the insect.

6.5 Conclusions and perspectives

To our knowledge, this study provides the first experimental evidence that *Lagria villosa* beetles can transmit mutualistic *Burkholderia* horizontally. It has been shown that horizontally transmitted symbionts are probably acquired at the larval stage and can be integrated into the newly formed symbiotic organs of the female adult beetles. The specific mechanisms by which the symbionts are acquired and potentially relocated remains to be tested in future experiments. However, it is likely that the symbiont integration from larval to adult stage is regulated similarly to the vertical transmission route (Buchner, 1965). Thus, whether the newly horizontally acquired

symbionts can be transmitted to next generations by vertical transmission remains to be tested as well.

Moreover, the beetles are capable of transmiting their symbionts to soybean plants, where they can reproduce, spread systemically and consequently have the potential to manipulate the host plant fitness. This might facilitate herbivory by the beetle, yet this remains to be assessed experimentally. Nevertheless, the symbiotic *B. gladioli* strain A isolated from *L. villosa* had no drastic but weakly pathogenic consequences in soybean plants. Contrastingly, cotyledon assays showed a defensive response by means of glyceollin production against *B.gladioli* strain A, suggesting that the symbiotic strain was recognized by the plants in early stages as a pathogen. It is unknown, however, if the symbionts could have a beneficially potential to protect the host plants against parasites. That would also be interesting to examine in future studies.

Further examinations with the two isolated morphotypes of *B. gladioli* strain A regarding metabolic profiles, biofilm formation traits and antibiotic susceptibility or virulence properties should be also done in future. In addition, cultivating and characterizing other symbiotic *B. gladioli* strains from lagriid beetles would be very useful for further experiments. Also, it would be interesting to identify the bacterial consortium of the reinfected aposymbiotic larvae (R-Nat), which were reported in this study and occasionally include bacteria different to *Burkholderia*. This would give additional insights on how symbionts are specialized to the insect host and how they compete against each other.

It finally seems as if the bacterial symbionts would be the evolutionary winners of the insect-symbiont-plant interaction. They are able to switch between different host lifestyles, where they can persist and reproduce. It is very likely that the insect mutualists evolved from plant parasitic bacteria. How symbiont recognition and selection has to be controlled by the insect and plant host immune system is very important for understanding interaction processes and has to be subsequently investigated in the future.

References

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R., and Stahl, D. A. (1990). COMBINATION OF 16S RIBOSOMAL-RNA-TARGETED OLIGONUCLEOTIDE PROBES WITH FLOW-CYTOMETRY FOR ANALYZING MIXED MICROBIAL-POPULATIONS. *Applied and Environmental Microbiology, 56*(6), 1919-1925.

Andolfi, A., Cimmino, A., Cantore, P. L., Iacobellis, N. S., and Evidente, A. (2008). Bioactive and structural metabolites of pseudomonas and burkholderia species causal agents of cultivated mushrooms diseases. *Perspectives in medicinal chemistry, 2*, 81-112.

Angus, A. A., Agapakis, C. M., Fong, S., Yerrapragada, S., Estrada-de los Santos, P., Yang, P., . . . Hirsch, A. M. (2014). Plant-Associated Symbiotic Burkholderia Species Lack Hallmark Strategies Required in Mammalian Pathogenesis. *Plos One, 9*(1). doi: 10.1371/journal.pone.0083779

Attafuah, A., and Bradbury, J. F. (1989). PSEUDOMONAS-ANTIMICROBICA, A NEW SPECIES STRONGLY ANTAGONISTIC TO PLANT-PATHOGENS. *Journal of Applied Bacteriology, 67*(6), 567-573. doi: 10.1111/j.1365-2672.1989.tb02529.x

Avery, S. V. (2006). Microbial cell individuality and the underlying sources of heterogeneity. *Nature Reviews Microbiology, 4*(8), 577-587. doi: 10.1038/nrmicro1460

Azeredo, E. H., and Rodrigues Cassino, P. C. (2004). BIOECOLOGIA E EFEITOS TRÓFICOS SOBRE Lagria villosa (FABRICIUS, 1783) (COLEOPTERA: LAGRIIDAE) EM ÁREAS DE BATATA, Solanum tuberosum. *L. Agronomia, 28*, 52-56.

Bary, A. d. (1879). *Die Erscheinung der Symbiose : Vortrag*. Strassburg: Verlag von Karl J. Trübner.

Baumann, P., Moran, N. A., and Baumann, L. (2006). *Bacteriocyte-Associated Endosymbionts of Insects*. New York: Springer.

Becerra, J. X., Venable, G. X., and Saeidi, V. (2015). Wolbachia-Free Heteropterans Do Not Produce Defensive Chemicals or Alarm Pheromones. *Journal of Chemical Ecology, 41*(7), 593-601. doi: 10.1007/s10886-015-0596-4

Bernabeu, P. R., Pistorio, M., Torres-Tejerizo, G., Estrada-De los Santos, P., Galar, M. L., Boiardi, J. L., and Luna, M. F. (2015). Colonization and plant growth-promotion of tomato by Burkholderia tropica. *Scientia Horticulturae, 191*, 113-120. doi: 10.1016/j.scienta.2015.05.014

Bourtzis, K., and Miller, T. A. (2009). *Insect symbiosis. Vol. 3 Vol. 3*. Boca Raton: CRC Press.

Boyanton, B. L., Noroski, L. M., Reddy, H., Dishop, M. K., Hicks, M. J., Versalovic, J., and Moylett, E. H. (2005). Burkholderia gladioli osteomyelitis in association with chronic granulomatous disease: Case report and review. *Pediatric Infectious Disease Journal, 24*(9), 837-839. doi: 10.1097/01.inf.0000177285.44374.dc

Breznak, J. A., and Brune, A. (1994). ROLE OF MICROORGANISMS IN THE DIGESTION OF LIGNOCELLULOSE BY TERMITES. *Annual Review of Entomology, 39*, 453-487. doi: 10.1146/annurev.en.39.010194.002321

Bright, M., and Bulgheresi, S. (2010). A complex journey: transmission of microbial symbionts. *Nature Reviews Microbiology, 8*(3), 218-230. doi: 10.1038/nrmicro2262

Brownlie, J. C., and Johnson, K. N. (2009). Symbiont-mediated protection in insect hosts. *Trends in Microbiology, 17*(8), 348-354. doi: 10.1016/j.tim.2009.05.005

Bruegger, B. B., and Keen, N. T. (1979). SPECIFIC ELICITORS OF GLYCEOLLIN ACCUMULATION IN THE PSEUDOMONAS-GLYCINEA-SOYBEAN HOST-PARASITE SYSTEM. *Physiological Plant Pathology, 15*(1), 43-51. doi: 10.1016/0048-4059(79)90038-9

Buchner, P. (1965). *Endosymbiosis of animals with plant microorganisms*. New York: Interscience, Inc.

Burkholder, W. H. (1950). SOUR SKIN, A BACTERIAL ROT OF ONION BULBS. *Phytopathology, 40*(1), 115-117.

Cardoza, Y. J., Klepzig, K. D., and Raffa, K. F. (2006). Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. *Ecological Entomology, 31*(6), 636-645. doi: 10.1111/j.1365-2311.2006.00829.x

Carlier, A. L., and Eberl, L. (2012). The eroded genome of a Psychotria leaf symbiont: hypotheses about lifestyle and interactions with its plant host. *Environmental Microbiology, 14*(10), 2757-2769. doi: 10.1111/j.1462-2920.2012.02763.x

Carpentieri-Pipolo, V., Mandarino, J. M. G., Carrao-Panizzi, M. C., Souza, A., and Kikuchi, A. (2005). Association of isoflavonoids with the incompatible response of soybean roots to Meloidogyne incognita race 3. *Nematropica, 35*(2), 103-110.

Caspi-Fluger, A., Inbar, M., Mozes-Daube, N., Katzir, N., Portnoy, V., Belausov, E., . . . Zchori-Fein, E. (2012). Horizontal transmission of the insect symbiont Rickettsia is plant-mediated. *Proceedings of the Royal Society B-Biological Sciences, 279*(1734), 1791-1796. doi: 10.1098/rspb.2011.2095

Chakravarthy, S., Velasquez, A. C., and Martin, G. B. (2009). *Assay for pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) in plants.* (; Research Support, U.S. Gov't, Non-P.H.S.; Video-Audio Media). Retrieved from <Go to ISI>://MEDLINE:19741586 (31)

Chang, J. H., Desveaux, D., and Creason, A. L. (2014). The ABCs and 123s of Bacterial Secretion Systems in Plant Pathogenesis. In N. K. VanAlfen (Ed.), *Annual Review of Phytopathology, Vol 52* (Vol. 52, pp. 317-345).

Chen, R., Barphagha, I. K., Karki, H. S., and Ham, J. H. (2012). Dissection of Quorum-Sensing Genes in Burkholderia glumae Reveals Non-Canonical Regulation and the New Regulatory Gene tofM for Toxoflavin Production. *Plos One, 7*(12). doi: 10.1371/journal.pone.0052150

Chowdhury, P. R., and Heinemann, J. A. (2006). The general secrotory pathway of Burkholderia gladioli pv. agaricicola BG164R is necessary for for cavity disease in white button mushrooms. *Applied and Environmental Microbiology, 72*(5), 3558-3565. doi: 10.1128/aem.72.5.3558-3565.2006

Coenye, T., Holmes, B., Kersters, K., Govan, J. R. W., and Vandamme, P. (1999). Burkholderia cocovenenans (van Damme et al. 1960) Gillis et al, 1995 and Burkholderia vandii Urakami et al. 1994 are junior synonyms of Burkholderia gladioli (Severini 1913) Yabuuchi et al. 1993 and Burkholderia plantarii (Azegami et al. 1987) Urakami et al 1994 respectively. *International Journal of Systematic Bacteriology, 49*, 37-42.

Coenye, T., and LiPuma, J. J. (2003). Molecular epidemiology of Burkholderia species. *Frontiers in Bioscience, 8*, E55-E67. doi: 10.2741/937

Coenye, T., and Vandamme, P. (2003). Diversity and significance of Burkholderia species occupying diverse ecological niches. *Environmental Microbiology, 5*(9), 719-729. doi: 10.1046/j.1462-2920.2003.00471.x

Currie, C. R., Scott, J. A., Summerbell, R. C., and Malloch, D. (1999). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature, 398*(6729), 701-704. doi: 10.1038/19519

Dale, C., Beeton, M., Harbison, C., Jones, T., and Pontes, M. (2006). Isolation, pure culture, and characterization of "Candidatus Arsenophonus arthropodicus," an intracellular secondary endosymbiont from the hippoboscid louse fly Pseudolynchia canariensis. *Applied and Environmental Microbiology, 72*(4), 2997-3004. doi: 10.1128/aem.74.4.2997-3004.2006

Darvill, A. G., and Albersheim, P. (1984). PHYTOALEXINS AND THEIR ELICITORS A DEFENSE AGAINST MICROBIAL INFECTION IN PLANTS *Briggs, W. R.* (pp. 243-276).

Delalibera, I., Jr., Vasanthakumar, A., Burwitz, B. J., Schloss, P. D., Klepzig, K. D., Handelsman, J., and Raffa, K. F. (2007). Composition of the bacterial community in the gut of the pine engraver, Ips pini (Say) (Coleoptera) colonizing red pine. *Symbiosis, 43*(2), 97-104.

Dobosy, J. R., Rose, S. D., Beltz, K. R., Rupp, S. M., Powers, K. M., Behlke, M. A., and Walder, J. A. (2011). RNase H-dependent PCR (rhPCR): improved specificity and single nucleotide polymorphism detection using blocked cleavable primers. *Bmc Biotechnology, 11*. doi: 10.1186/1472-6750-11-80

Dong, X. N. (1998). SA, JA, ethylene, and disease resistance in plants. *Current Opinion in Plant Biology, 1*(4), 316-323. doi: 10.1016/1369-5266(88)80053-0

Douglas, A. E. (2009). The microbial dimension in insect nutritional ecology. *Functional Ecology, 23*(1), 38-47. doi: 10.1111/j.1365-2435.2008.01442.x

Dursun, A., Zenciroglu, A., Karagol, B. S., Hakan, N., Okumus, N., Gol, N., and Tanir, G. (2012). Burkholderia gladioli sepsis in newborns. *European Journal of Pediatrics, 171*(10), 1503-1509. doi: 10.1007/s00431-012-1756-y

Ebert, D. (2013). The Epidemiology and Evolution of Symbionts with Mixed-Mode Transmission. In D. J. Futuyma (Ed.), *Annual Review of Ecology, Evolution, and Systematics, Vol 44* (Vol. 44, pp. 623-+).

Edwards, R. (1977). LAGRIA-VILLOSA COLEOPTERA TENEBRIONIDAE AN AFRICAN BEETLE ESTABLISHED IN BRAZIL. *Entomologist's Monthly Magazine, 113*(1360-1363), 202.

El-shakh, A. S. A., Kakar, K. U., Wang, X., Almoneafy, A. A., Ojaghian, M. R., Li, B., . . . Xie, G.-l. (2015). Controlling bacterial leaf blight of rice and enhancing the plant growth with endophytic and rhizobacterial Bacillus strains. *Toxicological and Environmental Chemistry, 97*(6), 766-785. doi: 10.1080/02772248.2015.1066176

Elshafie, H. S., Camele, I., Racioppi, R., Scrano, L., Iacobellis, N. S., and Bufo, S. A. (2012). In Vitro Antifungal Activity of Burkholderia gladioli pv. agaricicola against Some Phytopathogenic Fungi. *International Journal of Molecular Sciences, 13*(12), 16291-16302. doi: 10.3390/ijms131216291

Ettinger, C. L., Shehata, H. R., Johnston-Monje, D., Raizada, M. N., and Eisen, J. A. (2015). Draft Genome Sequence of Burkholderia gladioli Strain UCD-UG_CHAPALOTE (Phylum Proteobacteria). *Genome announcements, 3*(1). doi: 10.1128/genomeA.01462-14

Fett, W. F., and Jones, S. B. (1984). STRESS METABOLITE ACCUMULATION, BACTERIAL-GROWTH AND BACTERIAL IMMOBILIZATION DURING HOST AND NONHOST RESPONSES OF SOYBEAN TO BACTERIA. *Physiological Plant Pathology, 25*(3), 277-296. doi: 10.1016/0048-4059(84)90036-5

Fiori, M., Ligios, V., and Schiaffino, A. (2011). Identification and characterization of Burkholderia isolates obtained from bacterial rot of saffron (Crocus sativus L.) grown in Italy. *Phytopathologia Mediterranea, 50*(3), 450-461.

Flórez, L. V., Biedermann, P. H. W., Engl, T., and Kaltenpoth, M. (2015). Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. *Natural Product Reports, 32*(7), 904-936. doi: 10.1039/c5np00010f

Flórez, L. V., Gaube, P., Ross, C., Scherlach, K., Rodrigues, A., Hertweck, C., and Kaltenpoth, M. (in preparation). working title: Evolution of a protective insect mutualist from a plant pathogen.

Flórez, L. V., and Kaltenpoth, M. (in preparation). working title: Strain diversity and dynamics of symbiotic Burkholderia in a lagriid beetle.

Foley, P. L., LiPuma, J. J., and Feldman, S. H. (2004). Outbreak of otitis media caused by Burkholderia gladioli infection in immunocompromised mice. *Comparative Medicine, 54*(1), 93-99.

Fory, P. A., Triplett, L., Ballen, C., Abello, J. F., Duitama, J., Aricapa, M. G., . . . Mosquera, G. M. (2014). Comparative Analysis of Two Emerging Rice Seed Bacterial Pathogens. *Phytopathology, 104*(5), 436-444. doi: 10.1094/phyto-07-13-0186-r

Frago, E., Dicke, M., and Godfray, H. C. J. (2012). Insect symbionts as hidden players in insect-plant interactions. *Trends in Ecology & Evolution, 27*(12), 705-711. doi: 10.1016/j.tree.2012.08.013

Friesen, M. L., Porter, S. S., Stark, S. C., von Wettberg, E. J., Sachs, J. L., and Martinez-Romero, E. (2011). Microbially Mediated Plant Functional Traits. In D. J. Futuyma, H. B. Shaffer, & D. Simberloff (Eds.), *Annual Review of Ecology, Evolution, and Systematics, Vol 42* (Vol. 42, pp. 23-46).

Fukatsu, T., and Hosokawa, T. (2002). Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, Megacopta punctatissima. *Applied and Environmental Microbiology, 68*(1), 389-396. doi: 10.1128/aem.68.1.389-396.2002

Furuya, N., Iiyama, K., Shiozaki, N., and Matsuyama, N. (1997a). Phytotoxin produced by Burkholderia gladioli. *Journal of the Faculty of Agriculture Kyushu University, 42*(1-2), 33-37.

Furuya, N., Iiyama, K., Ueda, Y., and Matsuyama, N. (1997b). Reaction of tobacco and rice leaf tissue infiltrated with Burkholderia glumae or B-gladioli. *Journal of the Faculty of Agriculture Kyushu University, 42*(1-2), 43-51.

Futahashi, R., Tanaka, K., Tanahashi, M., Nikoh, N., Kikuchi, Y., Lee, B. L., and Fukatsu, T. (2013). Gene Expression in Gut Symbiotic Organ of Stinkbug Affected by Extracellular Bacterial Symbiont. *Plos One, 8*(5). doi: 10.1371/journal.pone.0064557

Gambetta, G. A., Fei, J., Rost, T. L., and Matthews, M. A. (2007). Leaf scorch symptoms are not correlated with bacterial populations during Pierce's disease. *Journal of Experimental Botany, 58*(15-16), 4037-4046. doi: 10.1093/jxb/erm260

Garcia, J. R., Laughton, A. M., Malik, Z., Parker, B. J., Trincot, C., Chiang, S. S. L., . . . Gerardo, N. M. (2014). Partner associations across sympatric broad-

headed bug species and their environmentally acquired bacterial symbionts. *Molecular Ecology, 23*(6), 1333-1347. doi: 10.1111/mec.12655

Garcia, M. A., and Pierozzi Junior, I. (1982). Aspectos da biologia e ecologia de Lagria villosa Fabricius, 1781 (Coleoptera, Lagriidae). *Rev. Brasil. Biol., 42*, 415-420.

Glick, B. R. (1995). THE ENHANCEMENT OF PLANT-GROWTH BY FREE-LIVING BACTERIA. *Canadian Journal of Microbiology, 41*(2), 109-117.

Gnanamanickam, S. S., and Immanuel, J. E. (2006). Epiphytic bacteria, their ecology and functions. *Plant-Associated Bacteria*, 131-153.

Gomez-Lama Cabanas, C., Schiliro, E., Valverde-Corredor, A., and Mercado-Blanco, J. (2014). The biocontrol endophytic bacterium Pseudomonas fluorescens PICF7 induces systemic defense responses in aerial tissues upon colonization of olive roots. *Frontiers in Microbiology, 5*. doi: 10.3389/fmicb.2014.00427

Gond, S. K., Bergen, M. S., Torres, M. S., and White, J. F., Jr. (2015). Endophytic Bacillus spp. produce antifungal lipopeptides and induce host defence gene expression in maize. *Microbiological Research, 172*, 79-87. doi: 10.1016/j.micres.2014.11.004

Gonella, E., Pajoro, M., Marzorati, M., Crotti, E., Mandrioli, M., Pontini, M., . . . Alma, A. (2015). Plant-mediated interspecific horizontal transmission of an intracellular symbiont in insects. *Scientific Reports, 5*. doi: 10.1038/srep15811

Grimault, V., and Prior, P. (1994). INVASIVENESS OF PSEUDOMONAS-SOLANACEARUM IN TOMATO, EGGPLANT AND PEPPER - A COMPARATIVE-STUDY. *European Journal of Plant Pathology, 100*(3-4), 259-267. doi: 10.1007/bf01876240

Hallmann, J., QuadtHallmann, A., Mahaffee, W. F., and Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology, 43*(10), 895-914.

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttila, A. M., Compant, S., Campisano, A., . . . Sessitsch, A. (2015). The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiology and Molecular Biology Reviews, 79*(3), 293-320. doi: 10.1128/mmbr.00050-14

Hill, B. L., and Purcell, A. H. (1995). MULTIPLICATION AND MOVEMENT OF XYLELLA-FASTIDIOSA WITHIN GRAPEVINE AND 4 OTHER PLANTS. *Phytopathology, 85*(11), 1368-1372. doi: 10.1094/Phyto-85-1368

Humphrey, P. T., Nguyen, T. T., Villalobos, M. M., and Whiteman, N. K. (2014). Diversity and abundance of phyllosphere bacteria are linked to insect herbivory. *Molecular Ecology, 23*(6), 1497-1515. doi: 10.1111/mec.12657

Hypsa, V. (1993). ENDOCYTOBIONTS OF TRIATOMA-INFESTANS - DISTRIBUTION AND TRANSMISSION. *Journal of Invertebrate Pathology, 61*(1), 32-38. doi: 10.1006/jipa.1993.1006

Hypsa, V., and Dale, C. (1997). In vitro culture and phylogenetic analysis of "Candidatus Arsenophonus triatominarum," an intracellular bacterium from the triatomine bug, Triatoma infestans. *International Journal of Systematic Bacteriology, 47*(4), 1140-1144.

Iiyama, K., Furuya, N., Takanami, Y., and Matsuyama, N. (1995). A role of phytotoxin in virulence of Pseudomonas glumae Kurita et Tabei. *Annals of the Phytopathological Society of Japan, 61*(5), 470-476.

Iiyama, K., Furuya, N., Ura, H., and Matsuyama, N. (1998). Role of phytotoxins in the pathogenesis of Burkholderia species. *Journal of the Faculty of Agriculture Kyushu University, 42*(3-4), 289-293.

Ingham, J. L., Keen, N. T., Mulheirn, L. J., and Lyne, R. L. (1981). INDUCIBLY-FORMED ISOFLAVONOIDS FROM LEAVES OF SOYBEAN. *Phytochemistry, 20*(4), 795-798. doi: 10.1016/0031-9422(81)85177-1

Inoue, T., Kitade, O., Yoshimura, T., and Yamaoka, I. (2000). Symbiotic associations with protists. *Termites: Evolution, Sociality, Symbioses, Ecology*, 275-288.

Itoh, H., Aita, M., Nagayama, A., Meng, X.-Y., Kamagata, Y., Navarro, R., . . . Kikuchi, Y. (2014). Evidence of Environmental and Vertical Transmission of Burkholderia Symbionts in the Oriental Chinch Bug, Cavelerius saccharivorus (Heteroptera: Blissidae). *Applied and Environmental Microbiology, 80*(19), 5974-5983. doi: 10.1128/aem.01087-14

Jeong, Y., Kim, J., Kim, S., Kang, Y., Nagamatsu, T., and Hwang, I. (2003). Toxoflavin produced by Burkholderia glumae causing rice grain rot is responsible for inducing bacterial wilt in many field crops. *Plant Disease, 87*(8), 890-895. doi: 10.1094/pdis.2003.87.8.890

Jha, G., Tyagi, I., Kumar, R., and Ghosh, S. (2015). Draft Genome Sequence of Broad-Spectrum Antifungal Bacterium Burkholderia gladioli Strain NGJ1, Isolated from Healthy Rice Seeds. *Genome announcements, 3*(4). doi: 10.1128/genomeA.00803-15

Johnston, P. R., and Rolff, J. (2015). Host and Symbiont Jointly Control Gut Microbiota during Complete Metamorphosis. *Plos Pathogens, 11*(11), e1005246-e1005246. doi: 10.1371/journal.ppat.1005246

Kaltenpoth, M., Gottler, W., Herzner, G., and Strohm, E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology, 15*(5), 475-479. doi: 10.1016/j.cub.2004.12.084

Keen, N. T. (1978). PHYTOALEXINS - EFFICIENT EXTRACTION FROM LEAVES BY A FACILITATED DIFFUSION TECHNIQUE. *Phytopathology, 68*(8), 1237-1239.

Kellner, R. L. L. (2002). Molecular identification of an endosymbiotic bacterium associated with pederin biosynthesis in Paederus sabaeus (Coleoptera : Staphylinidae). *Insect Biochemistry and Molecular Biology, 32*(4), 389-395. doi: 10.1016/s0965-1748(01)00115-1

Kennedy, M. P., Coakley, R. D., Donaldson, S. H., Aris, R. M., Hohneker, K., Olson, E. L., . . . Yankaskas, J. R. (2005). Burkholderia gladioli: Five year experience in a cystic fibrosis referral and lung transplantation center. *Chest, 128*(4), 152S-152S.

Kikuchi, Y. (2009). Endosymbiotic Bacteria in Insects: Their Diversity and Culturability. *Microbes and Environments, 24*(3), 195-204. doi: 10.1264/jsme2.ME09140S

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K., and Fukatsu, T. (2012). Symbiont-mediated insecticide resistance. *Proceedings of the National Academy of Sciences of the United States of America, 109*(22), 8618-8622. doi: 10.1073/pnas.1200231109

Kikuchi, Y., Hosokawa, T., and Fukatsu, T. (2007). Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Applied and Environmental Microbiology, 73*(13), 4308-4316. doi: 10.1128/aem.00067-07

Kikuchi, Y., Hosokawa, T., and Fukatsu, T. (2011a). An ancient but promiscuous host-symbiont association between Burkholderia gut symbionts and their heteropteran hosts. *Isme Journal, 5*(3), 446-460. doi: 10.1038/ismej.2010.150
Kikuchi, Y., Hosokawa, T., and Fukatsu, T. (2011b). Specific Developmental Window for Establishment of an Insect-Microbe Gut Symbiosis. *Applied and Environmental Microbiology, 77*(12), 4075-4081. doi: 10.1128/aem.00358-11
Kikuchi, Y., Meng, X. Y., and Fukatsu, T. (2005). Gut symbiotic bacteria of the genus Burkholderia in the broad-headed bugs Riptortus clavatus and Leptocorisa chinensis (Heteroptera : Alydidae). *Applied and Environmental Microbiology, 71*(7), 4035-4043. doi: 10.1128/aem.71.7.4035-4043.2005
Kikuchi, Y., and Yumoto, I. (2013). Efficient Colonization of the Bean Bug Riptortus pedestris by an Environmentally Transmitted Burkholderia Symbiont. *Applied and Environmental Microbiology, 79*(6), 2088-2091. doi: 10.1128/aem.03299-12
Kim, J., Kim, J. G., Kang, Y., Jang, J. Y., Jog, G. J., Lim, J. Y., . . . Hwang, I. (2004). Quorum sensing and the LysR-type transcriptional activator ToxR regulate toxoflavin biosynthesis and transport in Burkholderia glumae. *Molecular Microbiology, 54*(4), 921-934. doi: 10.1111/j.1365-2958.2004.04338.x
Kim, J. K., and Lee, B. L. (2015). SYMBIOTIC FACTORS IN Burkholderia ESSENTIAL FOR ESTABLISHING AN ASSOCIATION WITH THE BEAN BUG, Riptortus pedestris. *Archives of Insect Biochemistry and Physiology, 88*(1), 4-17. doi: 10.1002/arch.21218
Kim, J. K., Lee, J. B., Huh, Y. R., Jang, H. A., Kim, C. H., Yoo, J. W., and Lee, B. L. (2015). Burkholderia gut symbionts enhance the innate immunity of host Riptortus pedestris. *Developmental and Comparative Immunology, 53*(1), 265-269. doi: 10.1016/j.dci.2015.07.006
Kim, J. K., Son, D. W., Kim, C.-H., Cho, J. H., Marchetti, R., Silipo, A., . . . Lee, B. L. (2015). Insect Gut Symbiont Susceptibility to Host Antimicrobial Peptides Caused by Alteration of the Bacterial Cell Envelope. *Journal of Biological Chemistry, 290*(34), 21042-21053. doi: 10.1074/jbc.M115.651158
Kong, H., Shimosaka, M., Ando, Y., Nishiyama, K., Fujii, T., and Miyashita, K. (2001). Species-specific distribution of a modular family 19 chitinase gene in Burkholderia gladioli. *Fems Microbiology Ecology, 37*(2), 135-141. doi: 10.1016/s0168-6496(01)00154-4
Korves, T. M., and Bergelson, J. (2003). A developmental response to pathogen infection in Arabidopsis. *Plant Physiology, 133*(1), 339-347. doi: 10.1104/pp.103.027094
Kuklinsky-Sobral, H. L., Araujo, W. L., Mendes, R., Pizzirani-Kleiner, A. A., and Azevedo, J. L. (2005). Isolation and characterization of endophytic bacteria from soybean (Glycine max) grown in soil treated with glyphosate herbicide. *Plant and Soil, 273*(1-2), 91-99. doi: 10.1007/s11104-004-6894-1
Kurepin, L. V., Park, J. M., Lazarovits, G., and Huener, N. P. A. (2015). Involvement of plant stress hormones in Burkholderia phytofirmans-induced shoot and root growth promotion. *Plant Growth Regulation, 77*(2), 179-187. doi: 10.1007/s10725-015-0049-7
Lee, C. J., Lee, J. T., Kwon, J. H., Kim, B. C., and Park, W. (2005). Occurrence of bacterial soft rot of onion plants caused by Burkholderia gladioli pv. alliicola in Korea. *Australasian Plant Pathology, 34*(3), 287-292. doi: 10.1071/ap05024

Lee, J., Park, J., Kim, S., Park, I., and Seo, Y. S. (2016). Differential regulation of toxoflavin production and its role in the enhanced virulence of Burkholderia gladioli. *Mol Plant Pathol, 17*(1), 65-76. doi: 10.1111/mpp.12262

Lee, J. B., Byeon, J. H., Jang, H. A., Kim, J. K., Yoo, J. W., Kikuchi, Y., and Lee, B. L. (2015). Bacterial cell motility of Burkholderia gut symbiont is required to colonize the insect gut. *Febs Letters, 589*(19), 2784-2790. doi: 10.1016/j.febslet.2015.08.022

Login, F. H., Balmand, S., Vallier, A., Vincent-Monegat, C., Vigneron, A., Weiss-Gayet, M., . . . Heddi, A. (2011). Antimicrobial Peptides Keep Insect Endosymbionts Under Control. *Science, 334*(6054), 362-365. doi: 10.1126/science.1209728

Lund, S. T., Stall, R. E., and Klee, H. J. (1998). Ethylene regulates the susceptible response to pathogen infection in tomato. *Plant Cell, 10*(3), 371-382.

Lundgren, J. G., Lehman, R. M., and Chee-Sanford, J. (2007). Bacterial communities within digestive tracts of ground beetles (Coleoptera : Carabidae). *Annals of the Entomological Society of America, 100*(2), 275-282. doi: 10.1603/0013-8746(2007)100[275:bcwdto]2.0.co;2

Maeda, Y., Shinohara, H., Kiba, A., Ohnishi, K., Furuya, N., Kawamura, Y., . . . Hikichi, Y. (2006). Phylogenetic study and multiplex PCR-based detection of Burkholderia plantarii, Burkholderia glumae and Burkholderia gladioli using gyrB and rpoD sequences. *International Journal of Systematic and Evolutionary Microbiology, 56*, 1031-1038. doi: 10.1099/ijs.0.64184-0

McInroy, J. A., and Kloepper, J. W. (1994). *STUDIES ON INDIGENOUS ENDOPHYTIC BACTERIA OF SWEET CORN AND COTTON*. Weinheim: V C H Verlagsgesellschaft.

McInroy, J. A., and Kloepper, J. W. (1995). SURVEY OF INDIGENOUS BACTERIAL ENDOPHYTES FROM COTTON AND SWEET CORN. *Plant and Soil, 173*(2), 337-342. doi: 10.1007/bf00011472

Mehndiratta, M., Palanichamy, J. K., Ramalingam, P., Pal, A., Das, P., Sinha, S., and Chattopadhyay, P. (2008). Fluorescence acquisition during hybridization phase in quantitative real-time PCR improves specificity and signal-to-noise ratio. *Biotechniques, 45*(6), 625-+. doi: 10.2144/000112994

Mithofer, A., Wanner, G., and Boland, W. (2005). Effects of feeding Spodoptera littoralis on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiology, 137*(3), 1160-1168. doi: 10.1104/pp.104.054460

Moebius, N., Ross, C., Scherlach, K., Rohm, B., Roth, M., and Hertweck, C. (2012). Biosynthesis of the Respiratory Toxin Bongkrekic Acid in the Pathogenic Bacterium Burkholderia gladioli. *Chemistry & Biology, 19*(9), 1164-1174. doi: 10.1016/j.chembiol.2012.07.022

Mohr, P. G., and Cahill, D. M. (2001). Relative roles of glyceollin, lignin and the hypersensitive response and the influence of ABA in compatible and incompatible interactions of soybeans with Phytophthora sojae. *Physiological and Molecular Plant Pathology, 58*(1), 31-41. doi: 10.1006/pmpp.2000.0306

Montero, G. A., Carignano, M., Fernandez, C., Lietti, M. M., and Vignarolli, L. A. (2010). Defoliación temprana en cultivos de colza del centro-sur de Santa Fe. *Agromensajes, 29*.

Montero, G. A., Vignarolli, L., and Denoia, J. (2002). OTRO COLEÓPTERO CAUSA DAÑOS EN CULTIVOS DE SOJA EN SISTEMAS DE SIEMBRA DIRECTA. *Secretaría de extensión, FCA-UNR*.

Moran, N. A., Degnan, P. H., Santos, S. R., Dunbar, H. E., and Ochman, H. (2005). The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes. *Proceedings of the National Academy of Sciences of the United States of America, 102*(47), 16919-16926. doi: 10.1073/pnas.0507029102

Moran, N. A., McCutcheon, J. P., and Nakabachi, A. (2008). Genomics and Evolution of Heritable Bacterial Symbionts *Annual Review of Genetics* (Vol. 42, pp. 165-190). Palo Alto: Annual Reviews.

Moran, N. A., Russell, J. A., Koga, R., and Fukatsu, T. (2005). Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. *Applied and Environmental Microbiology, 71*(6), 3302-3310. doi: 10.1128/aem.71.6.3302-3310.2005

Mortensen, N. P., Fowlkes, J. D., Sullivan, C. J., Allison, D. P., Larsen, N. B., Molin, S., and Doktycz, M. J. (2009). Effects of Colistin on Surface Ultrastructure and Nanomechanics of Pseudomonas aeruginosa Cells. *Langmuir, 25*(6), 3728-3733. doi: 10.1021/la803898g

Mousa, W. K., Shearer, C. R., Limay-Rios, V., Zhou, T., and Raizada, M. N. (2015). Bacterial endophytes from wild maize suppress Fusarium graminearum in modern maize and inhibit mycotoxin accumulation. *Frontiers in Plant Science, 6*. doi: 10.3389/fpls.2015.00805

Muzammil, S., Graillon, C., Saria, R., Mathieu, F., Lebrihi, A., and Compant, S. (2014). The Saharan isolate Saccharothrix algeriensis NRRL B-24137 induces systemic resistance in Arabidopsis thalianaseedlings against Botrytis cinerea. *Plant and Soil, 374*(1-2), 423-434. doi: 10.1007/s11104-013-1864-0

Nadarasah, G., and Stavrinides, J. (2011). Insects as alternative hosts for phytopathogenic bacteria. *Fems Microbiology Reviews, 35*(3), 555-575. doi: 10.1111/j.1574-6976.2011.00264.x

Nandakumar, R., Rush, M. C., and Correa, F. (2007). Association of Burkholderia glumae and B-gladioli with panicle blight symptoms on rice in Panama. *Plant Disease, 91*(6), 767-767. doi: 10.1094/pdis-91-6-0767c

Nwachukwu, I. D., Luciano, F. B., and Udenigwe, C. C. (2013). The inducible soybean glyceollin phytoalexins with multifunctional health-promoting properties. *Food Research International, 54*(1), 1208-1216. doi: 10.1016/j.foodres.2013.01.024

Nybo, K. (2011). qPCR: avoiding signals in the no-template control. *Biotechniques, 50*(4), 213-+. doi: 10.2144/000113648

O'Donnell, P. J., Schmelz, E. A., Moussatche, P., Lund, S. T., Jones, J. B., and Klee, H. J. (2003). Susceptible to intolerance - a range of hormonal actions in a susceptible Arabidopsis pathogen response. *Plant Journal, 33*(2), 245-257. doi: 10.1046/j.1365-313X.2003.01619.x

Ohbayashi, T., Takeshita, K., Kitagawa, W., Nikoh, N., Koga, R., Meng, X.-Y., . . . Kikuchi, Y. (2015). Insect's intestinal organ for symbiont sorting. *Proceedings of the National Academy of Sciences of the United States of America, 112*(37), E5179-E5188. doi: 10.1073/pnas.1511454112

Oliver, K. M., and Moran, N. A. (2009). Defensive Symbionts in Aphids and Other Insects. In J. F. White & M. S. Torres (Eds.), *Defensive Mutualism in Microbial Symbiosis* (Vol. 27, pp. 129-147). Boca Raton: Crc Press-Taylor & Francis Group.

Oliver, K. M., Russell, J. A., Moran, N. A., and Hunter, M. S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps.

Proceedings of the National Academy of Sciences of the United States of America, 100(4), 1803-1807. doi: 10.1073/pnas.0335320100

Olivier-Espejel, S., Sabree, Z. L., Noge, K., and Becerra, J. X. (2011). Gut Microbiota in Nymph and Adults of the Giant Mesquite Bug (Thasus neocalifornicus) (Heteroptera: Coreidae) Is Dominated by Burkholderia Acquired De Novo Every Generation. *Environmental Entomology, 40*(5), 1102-1110. doi: 10.1603/en10309

Opelt, K., Berg, C., Schoenmann, S., Eberl, L., and Berg, G. (2007). High specificity but contrasting biodiversity of Sphagnum-associated bacterial and plant communities in bog ecosystems independent of the geographical region. *Isme Journal, 1*(6), 502-516. doi: 10.1038/ismej.2007.58

Oteino, N., Lally, R. D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K. J., and Dowling, D. N. (2015). Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. *Frontiers in Microbiology, 6*. doi: 10.3389/fmicb.2015.00745

Pacheco, J. M. (1978). Observações sobre a biologia de Lagria villosa (Coleoptera, Lagriidae): adultos e ovos. *Rev.bras.Ent., 22*, 105-108.

Pacheco, J. M., Matioli, J. C., and Muniz, J. M. (1976). "Lagria villosa" (Coleoptera: Lagridae), praga introduzida nas plantas cultivadas do Espirito Santo. *REUNIÃO ANUAL DA SBPC, 28*, 786-787.

Passam, H. C. (1975). EFFECTS OF BONGKREKIC ACID AND ATRACTYLOSIDE ON ADENINE-NUCLEOTIDE TRANSLOCATION IN PLANT MITOCHONDRIA. *Biochemical Society Transactions, 3*(1), 167-168.

Passari, A. K., Mishra, V. K., Gupta, V. K., Yadav, M. K., Saikia, R., and Singh, B. P. (2015). In Vitro and In Vivo Plant Growth Promoting Activities and DNA Fingerprinting of Antagonistic Endophytic Actinomycetes Associates with Medicinal Plants. *Plos One, 10*(9). doi: 10.1371/journal.pone.0139468

Paxton, J. D. (1981). PHYTOALEXINS - A WORKING RE-DEFINITION. *Phytopathologische Zeitschrift-Journal of Phytopathology, 101*(2), 106-109.

Paxton, J. D. (1995). *Soybean phytoalexins: Elicitation, nature, mode of action, and role*.

Piel, J. (2002). A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of Paederus beetles. *Proceedings of the National Academy of Sciences of the United States of America, 99*(22), 14002-14007. doi: 10.1073/pnas.222481399

Quadt-Hallmann, A., and Kloepper, J. W. (1996). Immunological detection and localization of the cotton endophyte Enterobacter asburiae JM22 in different plant species. *Canadian Journal of Microbiology, 42*(11), 1144-1154.

Quecine, M. C., Araujo, W. L., Rossetto, P. B., Ferreira, A., Tsui, S., Lacava, P. T., . . . Pizzirani-Kleiner, A. A. (2012). Sugarcane Growth Promotion by the Endophytic Bacterium Pantoea agglomerans 33.1. *Applied and Environmental Microbiology, 78*(21), 7511-7518. doi: 10.1128/aem.00836-12

Raso, A., Mascelli, S., Nozza, P., Ugolotti, E., Vanni, I., Capra, V., and Biassoni, R. (2011). Troubleshooting Fine-Tuning Procedures for qPCR System Design. *Journal of Clinical Laboratory Analysis, 25*(6), 389-394. doi: 10.1002/jcla.20489

Ratzka, C., Gross, R., and Feldhaar, H. (2013). Gene expression analysis of the endosymbiont-bearing midgut tissue during ontogeny of the carpenter ant

Camponotus floridanus. *Journal of Insect Physiology, 59*(6), 611-623. doi: 10.1016/j.jinsphys.2013.03.011

Ross, C., Opel, V., Scherlach, K., and Hertweck, C. (2014). Biosynthesis of antifungal and antibacterial polyketides by Burkholderia gladioli in coculture with Rhizopus microsporus. *Mycoses, 57*, 48-55. doi: 10.1111/myc.12246

Ross, J. P., Holland, S. M., Gill, V. J., Decarlo, E. S., and Gallin, J. I. (1995). SEVERE BURKHOLDERIA (PSEUDOMONAS) GLADIOLI INFECTION IN CHRONIC GRANULOMATOUS-DISEASE - REPORT OF 2 SUCCESSFULLY TREATED CASES. *Clinical Infectious Diseases, 21*(5), 1291-1293. doi: 10.1093/clinids/21.5.1291

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., and Dowling, D. N. (2008). Bacterial endophytes: recent developments and applications. *Fems Microbiology Letters, 278*(1), 1-9. doi: 10.1111/j.1574-6968.2007.00918.x

Sachs, J. L., Ehinger, M. O., and Simms, E. L. (2010). Origins of cheating and loss of symbiosis in wild Bradyrhizobium. *Journal of Evolutionary Biology, 23*(5), 1075-1089. doi: 10.1111/j.1420-9101.2010.01980.x

Saddler, G. S. (1994a). IMI DESCRIPTION OF FUNGI AND BACTERIA .1217. BURKHOLDERIA-GLADIOLI PV ALLIICOLA. *Mycopathologia, 128*(1), 55-56.

Saddler, G. S. (1994b). IMI DESCRIPTIONS OF FUNGI AND BACTERIA .1218. BURKHOLDERIA-GLADIOLI PV GLADIOLI. *Mycopathologia, 128*(1), 57-58.

Salem, H., Florez, L., Gerardo, N., and Kaltenpoth, M. (2015). An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proceedings of the Royal Society B-Biological Sciences, 282*(1804). doi: 10.1098/rspb.2014.2957

Salles, J. F., De Souza, F. A., and van Elsas, J. D. (2002). Molecular method to assess the diversity of Burkholderia species in environmental samples. *Applied and Environmental Microbiology, 68*(4), 1595-1603. doi: 10.1128/aem.68.4.1595-1603.2002

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA SEQUENCING WITH CHAIN-TERMINATING INHIBITORS. *Proceedings of the National Academy of Sciences of the United States of America, 74*(12), 5463-5467. doi: 10.1073/pnas.74.12.5463

Santos, A. V., Dillon, R. J., Dillon, V. M., Reynolds, S. E., and Samuels, R. I. (2004). Ocurrence of the antibiotic producing bacterium Burkholderia sp in colonies of the leaf-cutting ant Atta sexdens rubropilosa. *Fems Microbiology Letters, 239*(2), 319-323. doi: 10.1016/j.femsle.2004.09.005

Scarborough, C. L., Ferrari, J., and Godfray, H. C. J. (2005). Aphid protected from pathogen by endosymbiont. *Science, 310*(5755), 1781-1781. doi: 10.1126/science.1120180

Scott, J. J., Oh, D.-C., Yuceer, M. C., Klepzig, K. D., Clardy, J., and Currie, C. R. (2008). Bacterial protection of beetle-fungus mutualism. *Science, 322*(5898), 63-63. doi: 10.1126/science.1160423

Segonds, C., Clavel-Batut, P., Thouverez, M., Grenet, D., Le Coustumier, A., Plesiat, P., and Chabanon, G. (2009). Microbiological and Epidemiological Features of Clinical Respiratory Isolates of Burkholderia gladioli. *Journal of Clinical Microbiology, 47*(5), 1510-1516. doi: 10.1128/jcm.02489-08

Seo, Y.-S., Lim, J. Y., Park, J., Kim, S., Lee, H.-H., Cheong, H., . . . Hwang, I. (2015). Comparative genome analysis of rice-pathogenic Burkholderia

provides insight into capacity to adapt to different environments and hosts. *Bmc Genomics, 16*. doi: 10.1186/s12864-015-1558-5

Sequeira, L. (1985). Surface components involved in bacterial pathogen-plant host recognition. *Journal of cell science. Supplement, 2*, 301-316.

Setti de Liz, R., Anderson Guimaraes, J., Michereff Filho, I., and Pires de Mello Ribeiro, M. G. (2009). Manejo do Idiamim no Cultivo do Morangueiro. *Communicato Tecnico, 69*.

Severini, G. (1913). Una bacteriosi dell' Ixia maculata e del Gladiolus coluilli. *Ann.Bot.(Roma), 11*, 413-424.

Shimosaka, M., Fukumori, Y., Narita, T., Zhang, X. Y., Kodaira, R., Nogawa, M., and Okazaki, M. (2001). The bacterium Burkholderia gladioli strain CHB101 produces two different kinds of chitinases belonging to families 18 and 19 of the glycosyl hydrolases. *Journal of Bioscience and Bioengineering, 91*(1), 103-105. doi: 10.1263/jbb.91.103

Shin, J. H., Kim, S. H., Shin, M. G., Suh, S. P., Ryang, D. W., and Jeong, M. H. (1997). Bacteremia due to Burkholderia gladioli: Case report. *Clinical Infectious Diseases, 25*(5), 1264-1265. doi: 10.1086/516973

Shull, V. L., Vogler, A. P., Baker, M. D., Maddison, D. R., and Hammond, P. M. (2001). Sequence alignment of 18S ribosomal RNA and the basal relationships of Adephagan beetles: Evidence for monophyly of aquatic families and the placement of Trachypachidae. *Systematic Biology, 50*(6), 945-969. doi: 10.1080/106351501753462894

Simpson, I. N., Finlay, J., Winstanley, D. J., Dewhurst, N., Nelson, J. W., Butler, S. L., and Govan, J. R. W. (1994). MULTI-RESISTANCE ISOLATES POSSESSING CHARACTERISTICS OF BOTH BURKHOLDERIA-(PSEUDOMONAS)-CEPACIA AND BURKHOLDERIA-GLADIOLI FROM PATIENTS WITH CYSTIC-FIBROSIS. *Journal of Antimicrobial Chemotherapy, 34*(3), 353-361. doi: 10.1093/jac/34.3.353

Smith, J. M. (1989). EVOLUTION - GENERATING NOVELTY BY SYMBIOSIS. *Nature, 341*(6240), 284-285. doi: 10.1038/341284a0

Smits, W. K., Kuipers, O. P., and Veening, J. W. (2006). Phenotypic variation in bacteria: the role of feedback regulation. *Nature Reviews Microbiology, 4*(4), 259-271. doi: 10.1038/nrmicro1381

Snoeijers, S. S., Perez-Garcia, A., Joosten, M., and De Wit, P. (2000). The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogens. *European Journal of Plant Pathology, 106*(6), 493-506. doi: 10.1023/a:1008720704105

Sousa, A. M., Machado, I., and Pereira, M. O. (2011). Phenotypic switching: an opportunity to bacteria thrive. In A. Mendez-Vilas (Ed.), *Science against microbial pathogens: communicating current research and technological advances* (pp. 252-262). Spain: Formatex Research Center.

Spiers, A. J. (2007). Wrinkly-Spreader Fitness in the Two-Dimensional Agar Plate Microcosm: Maladaptation, Compensation and Ecological Success. *Plos One, 2*(8). doi: 10.1371/journal.pone.0000740

Spiers, A. J. (2014). A mechanistic explanation linking adaptive mutation, niche change, and fitness advantage for the wrinkly spreader. *International journal of evolutionary biology, 2014*, 675432-675432. doi: 10.1155/2014/675432

Spilman, T. J. (1978). Lagria villosa in Brazil, with new descriptions and illustrations of the larva and pupa (Coleoptera, Lagriidae). *Ciencia e Cultura (Sao Paulo), 30*(3), 342-347.

Stammer, H. J. (1929). Symbiosis in Lagrii-dae(Coleoptera). [Die Sym-biose der Lagriiden (Coleoptera)]. *Zeitschr Wiss Biol Abt a Zeitschr F Morph U Okol Tiere, 15*((1/2)), 1-34.

Stoyanova, M., Kizheva, Y., Chipeva, V., Bogatzevska, N., and Moncheva, P. (2011). PHYTOPATHOGENIC BURKHOLDERIA SPECIES IN BULB PLANTS IN BULGARIA. *Biotechnology & Biotechnological Equipment, 25*(3), 2477-2483. doi: 10.5504/bbeq.2011.0054

Stoyanova, M., Pavlina, I., Moncheva, P., and Bogatzevska, N. (2007). Biodiversity and incidence of Burkholderia species. *Biotechnology & Biotechnological Equipment, 21*(3), 306-310.

Subramanian, P., Kim, K., Krishnamoorthy, R., Sundaram, S., and Sa, T. (2015). Endophytic bacteria improve nodule function and plant nitrogen in soybean on co-inoculation with Bradyrhizobium japonicum MN110. *Plant Growth Regulation, 76*(3), 327-332. doi: 10.1007/s10725-014-9993-x

Sudakaran, S., Retz, F., Kikuchi, Y., Kost, C., and Kaltenpoth, M. (2015). Evolutionary transition in symbiotic syndromes enabled diversification of phytophagous insects on an imbalanced diet. *Isme Journal, 9*(12), 2587-2604.

Suzuki, F., Sawada, H., and Matsuda, I. (1998). Molecular characterization of toxoflavin biosynthesis-related gene in Pseudomonas (Burkholderia) glumae. *Annals of the Phytopathological Society of Japan, 64*(4), 276-281.

Tampakaki, A. P., Hatziloukas, E., and Panopoulos, N. J. (2009). Plant Pathogens, Bacteria. In M. Schaechter (Ed.), *Encyclopedia of microbiology* (3 ed., pp. 665-677). Amsterdam, Boston: Eslevier/ Academic Press.

Tayeb, L. A., Lefevre, M., Passet, V., Diancourt, L., Brisse, S., and Grimont, P. A. D. (2008). Comparative phylogenies of Burkholderia, Ralstonia, Comamonas, Brevundimonas and related organisms derived from rpoB, gyrB and rrs gene sequences. *Research in Microbiology, 159*(3), 169-177. doi: 10.1016/j.resmic.2007.12.005

Teixeira, L., Ferreira, A., and Ashburner, M. (2008). The Bacterial Symbiont Wolbachia Induces Resistance to RNA Viral Infections in Drosophila melanogaster. *Plos Biology, 6*(12), 2753-2763. doi: 10.1371/journal.pbio.1000002

Teo, I. A., Choi, J. W., Morlese, J., Taylor, G., and Shaunak, S. (2002). LightCycler qPCR optimisation for low copy number target DNA. *Journal of Immunological Methods, 270*(1), 119-133. doi: 10.1016/s0022-1759(02)00218-1

Tichopad, A., Bar, T., Pecen, L., Kitchen, R. R., Kubista, M., and Pfaffl, M. W. (2010). Quality control for quantitative PCR based on amplification compatibility test. *Methods, 50*(4), 308-312. doi: 10.1016/j.ymeth.2010.01.028

Tong, Y., Dou, L., and Wang, C. (2013). Peritonitis due to Burkholderia gladioli. *Diagnostic Microbiology and Infectious Disease, 77*(2), 174-175. doi: 10.1016/j.diagmicrobio.2013.06.010

Touceda-Gonzalez, M., Brader, G., Antonielli, L., Ravindran, V. B., Waldner, G., Friesl-Hanl, W., . . . Sessitsch, A. (2015). Combined amendment of immobilizers and the plant growth-promoting strain Burkholderia phytofirmans PsJN favours plant growth and reduces heavy metal uptake. *Soil Biology & Biochemistry, 91*, 140-150. doi: 10.1016/j.soilbio.2015.08.038

Tschinkel, W. R., and Doyen, J. T. (1980). COMPARATIVE ANATOMY OF THE DEFENSIVE GLANDS, OVIPOSITORS AND FEMALE GENITAL

TUBES OF TENEBRIONID BEETLES (COLEOPTERA). *International Journal of Insect Morphology & Embryology, 9*(5-6), 321-368. doi: 10.1016/0020-7322(80)90009-4

Tsiantos, J., and Stevens, W. A. (1986). The population dynamics of Corynebacterium michiganense pv. michiganense and other selected bacteria in tomato leaves. *Phytopathologia Mediterranea, 25*(1-3), 160-162.

Um, S., Fraimout, A., Sapountzis, P., Oh, D. C., and Poulsen, M. (2013). The fungus-growing termite Macrotermes natalensis harbors bacillaene-producing Bacillus sp that inhibit potentially antagonistic fungi. *Scientific Reports, 3*, 7. doi: 10.1038/srep03250

Ura, H., Furuya, N., Iiyama, K., Hidaka, M., Tsuchiya, K., and Matsuyama, N. (2006). Burkholderia gladioli associated with symptoms of bacterial grain rot and leaf-sheath browning of rice plants. *Journal of General Plant Pathology, 72*(2), 98-103. doi: 10.1007/s10327-005-0256-6

van Borm, S., Buschinger, A., Boomsma, J. J., and Billen, J. (2002). Tetraponera ants have gut symbionts related to nitrogen-fixing root-nodule bacteria. *Proceedings of the Royal Society B-Biological Sciences, 269*(1504), 2023-2027. doi: 10.1098/rspb.2002.2101

Vandamme, P. A., Johannes, A. G., Cox, H. C., and Berends, W. (1960). ON TOXOFLAVIN, THE YELLOW POISON OF PSEUDOMONAS COCOVENENANS. *Recueil Des Travaux Chimiques Des Pays-Bas-Journal of the Royal Netherlands Chemical Society, 79*(3), 255-267.

Vega, F. E., Pava-Ripoll, M., Posada, F., and Buyer, J. S. (2005). Endophytic bacteria in Coffea arabica L. *Journal of Basic Microbiology, 45*(5), 371-380. doi: 10.1002/jobm.200410551

Vidhyasekaran, P. (2008). *Fungal Pathogenesis in Plants and Crops: Molecular Biology and Host Defense Mechanisms, Second Edition*.

Villas Boas, G. L. (1982). Estudo da biologia e danos de Lagria villosa (Coleoptera lagriidae) em soja. *SEMINARIO NACIONAL DE PESQUISA DE SOJA, 2*(EMBRAPA-CNPSo, 1982), 83-91.

Wang, L.-T., Lee, F.-L., Tai, C.-J., and Kasai, H. (2007). Comparison of gyrB gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the Bacillus subtilis group. *International Journal of Systematic and Evolutionary Microbiology, 57*, 1846-1850. doi: 10.1099/ijs.0.64685-0

Weintraub, P. G., and Beanland, L. (2006). Insect vectors of phytoplasmas *Annual Review of Entomology* (Vol. 51, pp. 91-111).

Wen, Z., Yu, L., Yang, W., Wang, J., Zhao, J., Li, N., . . . Li, J. (2009). Detection of viral aerosols by use of real-time quantitative PCR. *Aerobiologia, 25*(2), 65-73. doi: 10.1007/s10453-009-9110-1

Xia, Y., DeBolt, S., Dreyer, J., Scott, D., and Williams, M. A. (2015). Characterization of culturable bacterial endophytes and their capacity to promote plant growth from plants grown using organic or conventional practices. *Frontiers in Plant Science, 6*. doi: 10.3389/fpls.2015.00490

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., . . . Arakawa, M. (1992). PROPOSAL OF BURKHOLDERIA GEN-NOV AND TRANSFER OF 7 SPECIES OF THE GENUS PSEUDOMONAS HOMOLOGY GROUP-II TO THE NEW GENUS, WITH THE TYPE SPECIES BURKHOLDERIA-CEPACIA (PALLERONI AND HOLMES 1981) COMB-NOV. *Microbiology and Immunology, 36*(12), 1251-1275.

Yamamoto, S., and Harayama, S. (1995). PCR AMPLIFICATION AND DIRECT SEQUENCING OF GYRB GENES WITH UNIVERSAL PRIMERS AND THEIR APPLICATION TO THE DETECTION AND TAXONOMIC ANALYSIS OF PSEUDOMONAS-PUTIDA STRAINS. *Applied and Environmental Microbiology, 61*(3), 1104-1109.

Yan, Q.-H., Zhou, J.-X., Li, H.-Z., Zhi, Q.-Q., Zhou, X.-P., and He, Z.-M. (2015). Coexistence of and interaction relationships between an aflatoxin-producing fungus and a bacterium. *Fungal Biology, 119*(7), 605-614. doi: 10.1016/j.funbio.2015.03.006

Yoneyama, K., Kono, Y., Yamaguchi, I., Horikoshi, M., and Hirooka, T. (1998). Toxoflavin is an essential factor for virulence of Burkholderia glumae causing rice seedling rot disease. *Annals of the Phytopathological Society of Japan, 64*(2), 91-96.

Zablotowicz, R. M., Tipping, E. M., Lifshitz, R., and Kloepper, J. W. (1991). Plant growth promotion mediated by bacterial rhizosphere colonizers. In D. Keister & P. Cregan (Eds.), *The Rhizosphere and Plant Growth* (Vol. 14, pp. 315-326): Springer Netherlands.

Zahringer, U., Schaller, E., and Grisebach, H. (1981). INDUCTION OF PHYTOALEXIN SYNTHESIS IN SOYBEAN - STRUCTURE AND REACTINS OF NATURALLY-OCCURRING AND ENZYMATICALLY PREPARED PRENYLATED PTEROCARPANS FROM ELICITOR-TREATED COTYLEDONS AND CELL-CULTURES OF SOYBEAN. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences, 36*(3-4), 234-241.

Zipfel, C. (2014). Plant pattern-recognition receptors. *Trends in Immunology, 35*(7), 345-351. doi: 10.1016/j.it.2014.05.004

List of abbreviations

®	registered trademark symbol
°C	degree Celsius
16S rRNA	16S ribosomal ribonucleic acid
ANOVA	analysis of variance
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CFU(s)	colony forming unit(s)
Cy3/Cy5	cyanine (fluorescent dye)
DAPI	4´,6-diamidino-2-pheylindole
DEPC	diethyl dicarbonate
dH_2O	destilled water
dNTP	deoxyribonucleid acid
EDTA	ethylenediamine tetraacetic acid
ESI	electrospray ionization
EtOH	ethanol
FISH	fluorescent in situ hybridization
fwd. (or) FW	forward
GABA	gamma-aminobutyric acid
GLMM	generalized linear mixed model
gyr B	gyrase B subunit gene
HCl	hydrogen chlorid
HPLC	high-performance liquid chromatography
KCl	potassium chloride
LB	Luriana Bertani
LC	liquid chromatography
M	molar
mAU	milli absorption units
MeOH	methanol
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
mM	milli molar

MS	mass spectrometry
NaClO	sodium chloride
NTC	no template control
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Q-TOF	quadrupole time-of-flight
rev. (or) RW	reverse
R-Nat	reinfected (with the) natural bacterial consortium
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sp. (plural spp.)	species
Taq	*Thermus aquaticus*
THP	3,6,9-trihydroxypterocarpan
Tris	tris(hydroxymethyl)aminomethane
U	unit(s)
UV-Vis	ultraviolet–visible

Supplemental Material

Table S1. Burkholderia presence in different treatments of *L. villosa* larvae and adults (from the same egg clutch), detected by FISH or diagnostic PCR.

	FISH		**diagnostic PCR**	
treatment	**larvae**	**adults**	**larvae**	**adults**
untreated control	1/1	1/1	-	4/5
reinfected (natural community)	0/1	0/1	1/1	0/2
aposymbiotic	0/1	0/1	0/6	1/5*

* positive band with very weak intensity

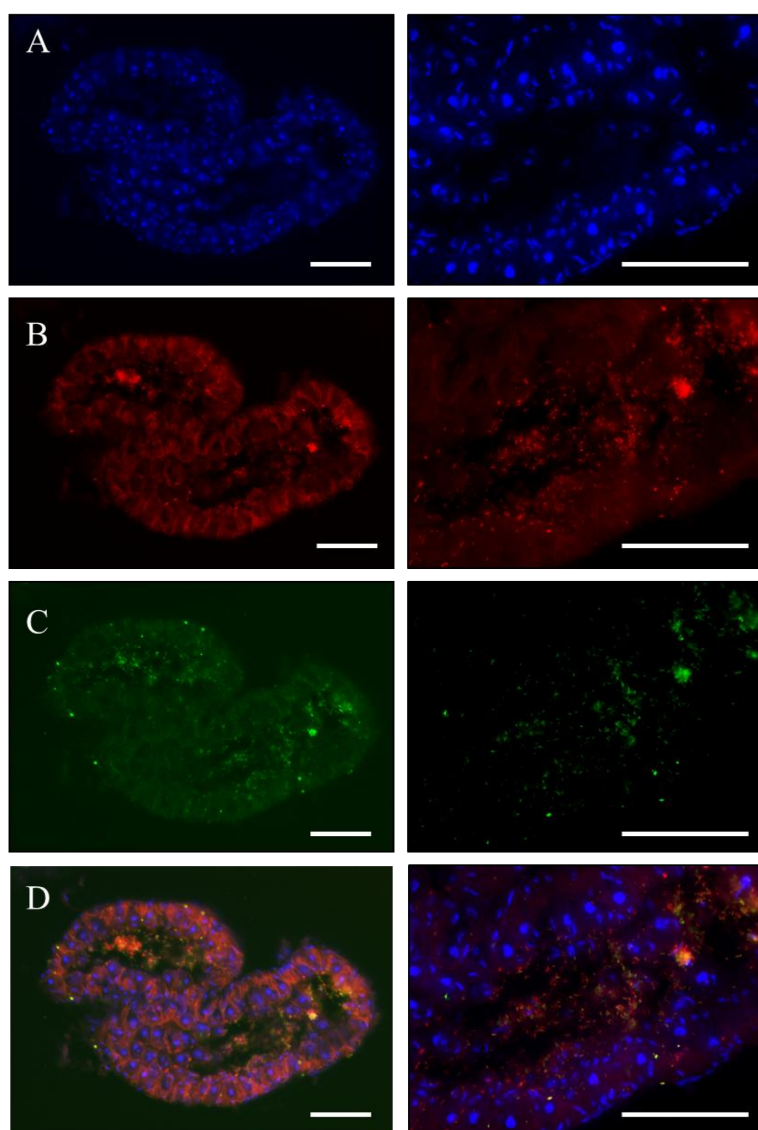

Figure S1. FISH on cross-sections (8 µm) showing the symbiont-harboring structures of previously aposymbiotic *Lagria villosa* exposed to *Burkholderia* on leaf litter. (**A**) Host nuclei cells are represented in blue (DAPI), (**B**) *Burkholderia* specific in red (Burk_16S-Cy3), (**C**) general eubacterial staining in green (EUB33-Cy5) and (**D**) the merge overlapping of channel Cy3 and Cy5 is emphasized in yellow. Scale bars represent 50 µm. Single channel images correspond to Figure 9A & B.

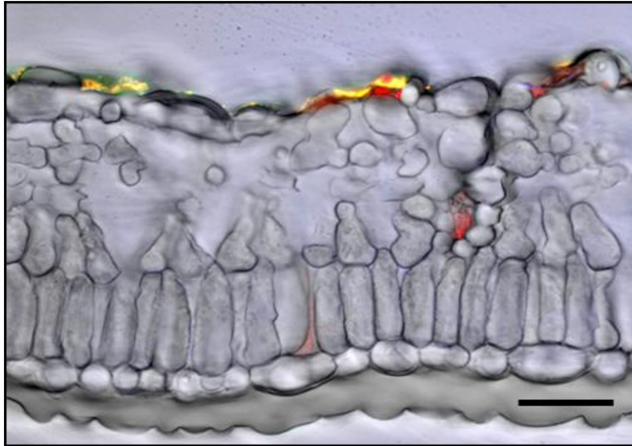

Figure S2. FISH on cross-section of soybean leaves infected with symbiotic *B. gladioli* strain A. The picture shows the merge of bright field microscopy, *Burkholderia* specific signals in red (Burk_16S-Cy3), eubacterial staining in green (EUB784-Cy5), and the merge overlapping of Cy3 and Cy5 is emphasized in yellow, indicating living bacterial cells on the leaf surface. The Scale bar represents 50 µm.

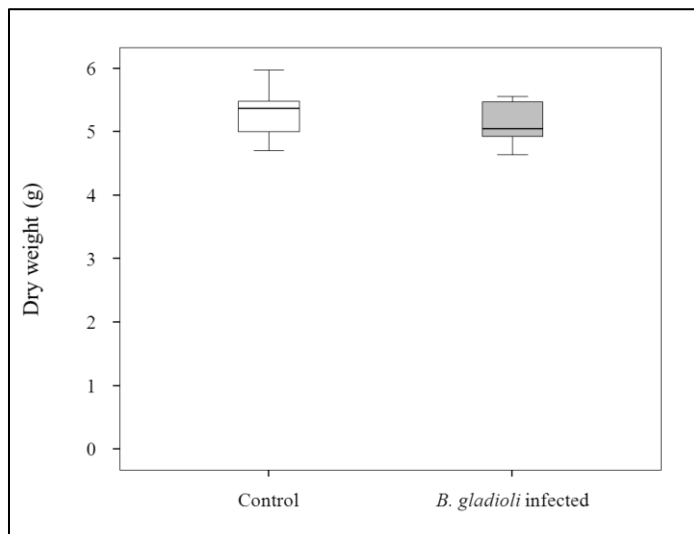

Figure S3. Dry weight of soybean plants (without roots), 38 days after *B. gladioli* strain A infection in comparison to control plants.

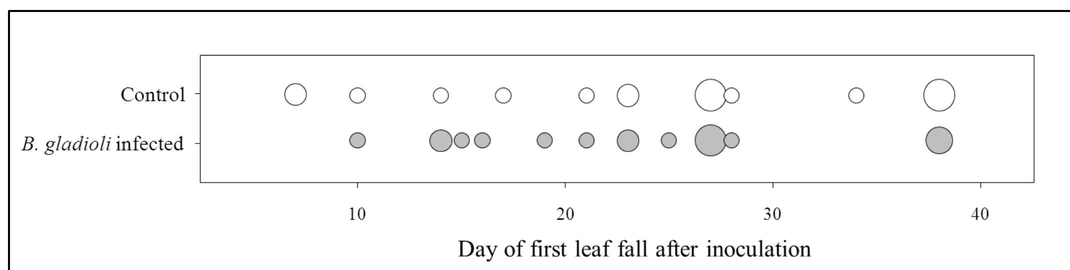

Figure S4. First leaf fall events of soybean plants infected with *B. gladioli* strain A and treated with sterile tap H_2O as a control. The radius is proportional to the no, of individuals that showed the first leaf loss at given time points.

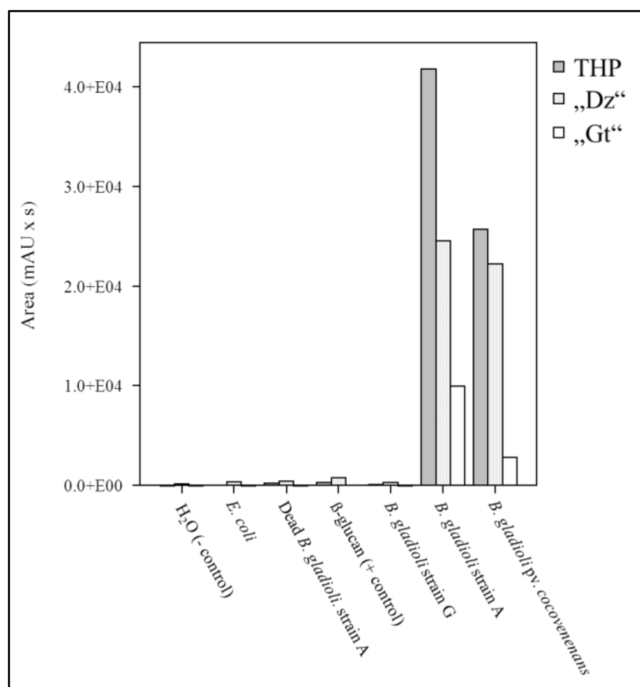

Figure S5. Relative amounts of THP (glycinol) and two unidentified substances, possibly daidzein ("Dz") and genistein ("Gt"), expressed as milli absorbance units for seconds (mAU x s) due to the lack of defined standards. Values were obtained from HPLC analysis results (Figure S7)

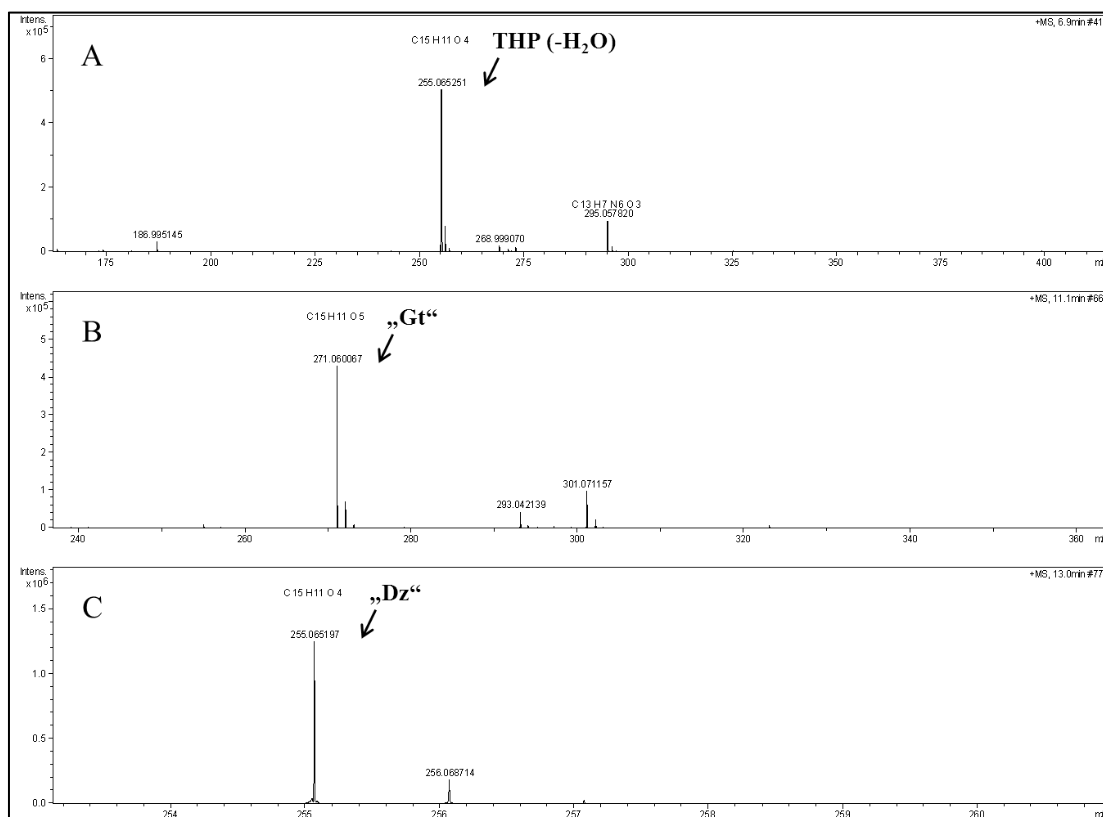

Figure S6. MS profiles of the cotyledon assay extracts. (**A**) Glycinol (THP -H20) and two unidentified substances, possibly (**B**) genistein ("Gt") and (**C**) daidzein ("Dz") are represented, respectively. Mass spectrometry was measured in positive ESI mode.

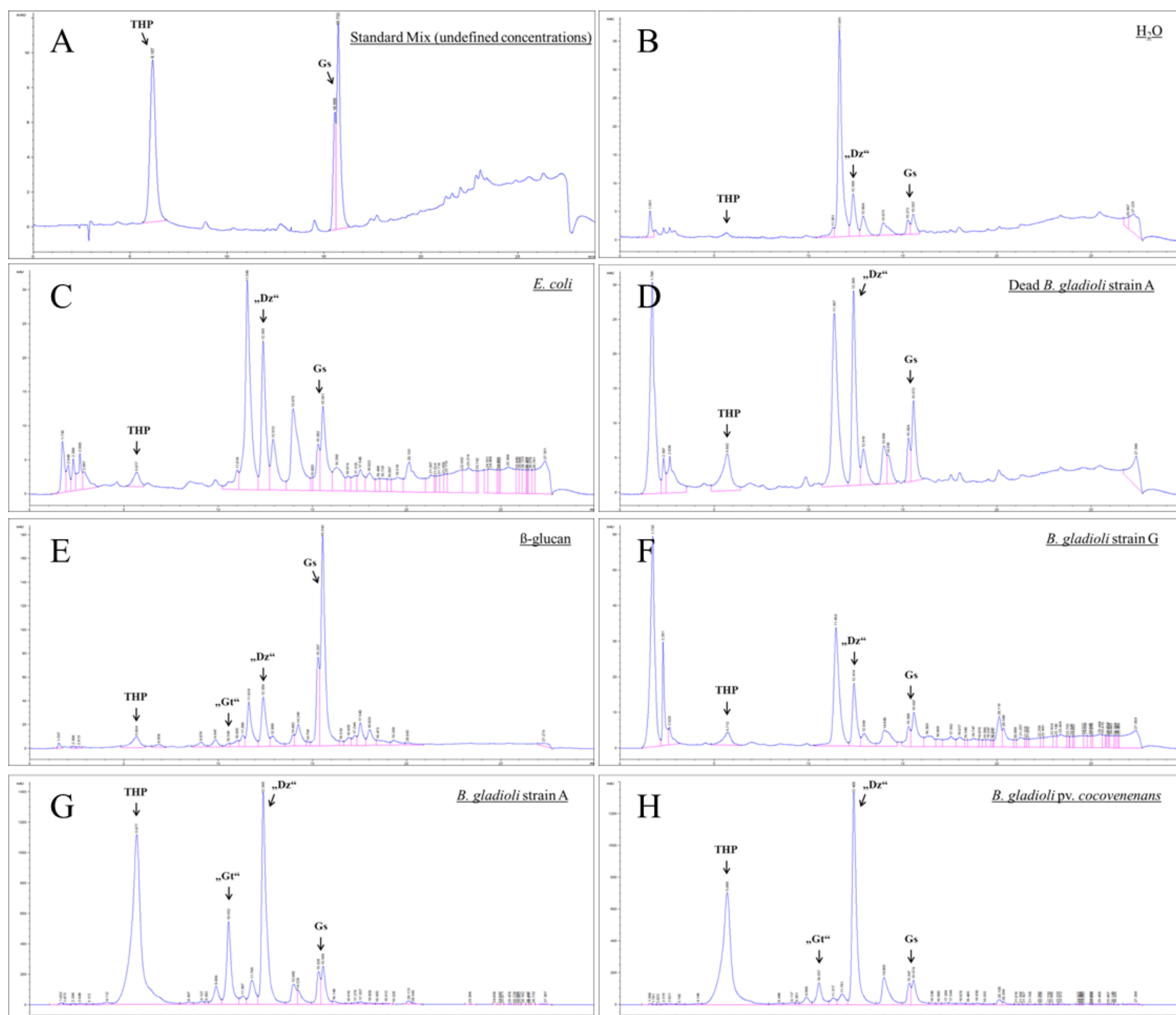

Figure S7. HPLC profiles of extracts from the cotyledon assay. Cotyledons were inoculated for 24 h with different treatments: (**B**) sterile tap H_2O, (**C**) *E. coli* K-12, (**D**) dead *B. gladioli* strain A from *L. villosa*, (**E**) ß-glucan, (**F**) *B. gladioli* strain G from *L. hirta*, (**G**) *B. gladioli* strain A from *L. villosa* and (**H**) *B. gladioli* pv. *cocovenenans.* (**A**) A mixture of undefined concentration of THP and glyceollin was used as a positive control for compound detection. Glyceollin isomers are represented by a double peak (Gs). Glycinol (THP) and two unidentified substances, likely daidzein ("Dz") and genistein ("Gt") are represented by a single peak (arrows).

Danksagung

Ich möchte zuallererst bei Martin Kaltenpoth und Laura Floréz für die Einführung in die Welt der Insektensymbiosen und für ihre exzellente Betreuung der Masterarbeit bedanken. Außerdem bedanke ich mich für das Vertrauen, welches in mich gesetzt wurde sowie für die zahlreichen Gespräche, Diskussionen und Anregungen.
Ganz besonders möchte ich Laura danken, dafür dass sie mich bei allem unterstützt hat und für ihre Herzlichkeit.
Martin bin ich sehr dankbar, für seine Ideen, für die Bereitstellung des Themas meiner Arbeit und vor allem für seine Begeisterungsfähigkeit für die Wissenschaft und für die erzielten Resultate, welche dank ihm auch auf mich übergesprungen ist.
Ich empfand mich zu jeder Zeit gut aufgehoben und beraten in der Max-Planck-Forschungsgruppe Insektensymbiosen. Ich möchte mich für das enorm produktive und positive Arbeitsklima, die freundschaftlichen Gespräche, verbrachten Stunden, gegessen Kuchen und wunderbaren unzähligen Stunden bei meinen Kollegen Aileen, Benni, Eric, Hassan, Melissa, Peter, Sailen, Shantanu, Thomas und Tobi bedanken.
Benjamin Weiss danke ich sehr für die Herstellung der FISH-Semidünnschnitte und für seine praktischen Ratschläge.
Dem Max-Planck-Institut für Chemische Ökologie danke ich für den Arbeitsplatz, die Bereitstellung der benötigten Materialen sowie für die wunderbare Arbeitsatmosphäre. Der Entomologie-Abteilung des Max-Planck-Instituts für Chemische Ökologie danke ich für das Benutzen des Nanodrop-Messgerätes sowie für das Sequenzieren meiner Proben.
Axel Mitthöfer danke ich für die Bereitstellung des Glyceollinstandards und der ß-glucan-Probe sowie für die Diskussionen und die Einführung in die Thematik der Pflanzenpathogenität.
Kerstin Ploß danke ich für die HPLC-MS-Analysen und die guten Gespräche.
Prof. Dr. Erika Kothe danke ich sehr, für die freundliche Übernahme der Zweitbegutachtung dieser Arbeit und für das vermittelte Wissen während meines Studiums.
Zuletzt danke ich meiner geliebten Sonja für ihre Liebe sowie auch für ihre Geduld und Unterstützung während meines Studiums.

Selbstständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe und alle Gedanken, die anderen wissenschaftlichen Arbeiten wörtlich oder sinngemäß entnommen wurden, kenntlich gemacht sind.

Seitens des Verfassers bestehen keine Einwände die vorliegende Arbeit für die öffentliche Benutzung im Universitätsarchiv zur Verfügung zu stellen.

PAUL GAUBE

Jena, den 29. Januar 2016