THE ENDOSYMBIOTIC BACTERIA OF THE SOUTH AFRICAN BIRD CHERRY-OAT APHID, *Rhopalosiphum padi*

by

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Submitted in fulfillment of the academic requirements for the degree of Master of Science (MSc)

Microbiology Discipline

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August 2018

As the candidate's supervisor I have/have not approved this thesis/dissertation for submission.

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PREFACE

The experimental work described in this dissertation was completed by Ms. Bongiwe Nhlapho while based at the Agricultural Research Council-Small Grain Institute, in Bethlehem, Free State, South Africa as a student registered under the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa. The research was financially supported by the National Research Foundation.

The contents of this dissertation have not been submitted for any degree or examination at any other institution and except where indicated in this dissertation, the results reported are due to my investigations.

Supervisor: Prof. Johnson Lin

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Date: 17 August 2018

DECLARATION-PLAGIARISM

I, Bongiwe N. Nhlapho, declare that:

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ABSTRACT

Cereal crops play a major role in human diet as staple foods, especially in developing countries. These crops are a part of a few edible crops that are widely cultivated globally. However, the production of these crops is constantly being put under strain by abiotic and biotic stresses in the environment, such as aphids. Aphids are the most important insects of cereal crops, not only causing damage through feeding. Aphids such as the bird cherry-oat aphid also transmit harmful plant viruses. They feed using piercing and sucking mouthparts that they insert into the plant while excreting saliva, which suppresses the plant's defence mechanism. The plant sap these insects feed on is rich in sugars and deficient in essential nutrients required for their optimal growth and reproduction processes. These insects live in symbiotic associations with endosymbiotic bacteria, which synthesise the deficient nutrients for the aphids. These bacteria also produce a chaperon protein that has been hypothesised to be involved in protecting the viruses they transmit from degradation. In South Africa, there is limited information about the endosymbiotic bacteria of the bird cherry-oat aphid. This aphid is estimated to cause substantial cereal crop yield losses through feeding, mostly through transmitting barley yellow dwarf viruses. Persistence of these pests may lead to a reduced harvest of these crops, which might result in a drastic rise in hunger and poverty and serious economic consequences. This study aimed to investigate the association between the bird cherry-oat aphid and its endosymbiotic bacteria, with the hope that the findings will give further understanding on how to manage this pest. Molecular biology techniques were employed to identify the endosymbiotic bacteria of the bird cherry-oat aphid using 16S rDNA. Once identified, the effects of two antibiotics on the survival and reproduction of bird cherry-oat aphids were compared using a flask method which was found to be superior in rearing aphids compared to an artificial diet. This study also assessed the ability of bird cherry-oat aphids to acquire *Hamiltonella defensa* secondary endosymbiont from infested rose grain aphid through a shared food source. The results obtained in this study show that the obligate primary endosymbiont, *Buchnera aphidicola*, was found across all the screened samples, while sporadic occurrence was observed for the secondary endosymbionts. In addition, this study also showed that in the absence of their primary endosymbiotic bacteria, bird cherry-oat aphids could not reproduce and though aphid death was not immediate, most of the aphids had died by the end of the experiment. Lastly, this study showed that secondary endosymbionts can be passed between aphids through a shared food source. The field provides crops with a vast number of microbes, which can be interchangeable between plants and aphids. However, the most essential microbe, *B. aphidicola*, uses the aphid as a host and controlling this endosymbiont might lead to a potential control measure for the bird cherry-oat aphid.

ACKNOWLEDGEMENTS

I would like to thank the following people for all their contributions in making this project a possibility:

- My supervisors, Prof. Johnson Lin and Dr. Scott Sydenham, for all the guidance from the beginning when the project was just an idea up to the last day when it all became a reality
- Dr. Goddy Prinsloo for providing the aphids and space to do all the experimental work
- Mr. Pinkie Hadebe for maintaining the aphids
- National Research Foundation for providing the funds for the project
- Lastly my loving family for being there and knowing they will always be there everytime I need them

I appreciate all of you dearly.

"What we do for ourselves dies with us-what we do for others remain and is immortal"

Albert pike

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CHAPTER1: GENERAL INTRODUCTION

There are over 50 000 edible plant species that have been identified, but only a few make a significant contribution to the human diet (Leef *et al*., 2004; Killian, 2012). Ten thousand belong to the cereal family, but only a few are being widely cultivated (Leef *et al*., 2004; Seck *et al*., 2012). Cereal crops are one of the major constituents of the starchy staples in the human diet and the primary source of dietary carbohydrates globally, especially in developing countries (Awika, 2011). These crops are staple foods of about 80% of the world's population, contributing about 47% in the African diet and 26% in that of the developed world (Leef *et al*., 2004; Awika, 2011, Seck *et al*., 2012). This clearly shows the significant role these crops play. However, the constant and continuous invasions by aphids on these crops, places them under serious threat.

Aphids are one of the most successful groups of insects, due to their reproductive and feeding capabilities, which involve very little movement (Breandle *et al*., 2003; Leroy *et al*., 2010; Ghaffar *et al*., 2014; Vereschagina and Gandrabur, 2014). An individual aphid is estimated to produce about 70 offspring in its lifespan, which is between 15-25 days (Ghaffar *et al*., 2014). They spend most of their lifespan probing and sucking on phloem-sap of cultivated crops, moving only when conditions become unfavourable, causing significant damage to cultivated crops thus making them the worst enemies of the agricultural industries (Breandle *et al*., 2003; Vereschagina and Gandrabur, 2014). The plants they feed on consist mainly of sugars and lack other essential nutrients required by the aphids, which they are unable to synthesize (Gomez-Valero *et al*., 2004; Tsuchida *et al*., 2014; Renoz *et al*., 2015; Zhang *et al*., 2015). Aphids are able to obtain these nutrients through the primary endosymbiotic bacteria that they house within their bacteriocyte cell structures (Breandle *et al*., 2003; Ateyyat, 2008; Koga *et al*., 2012). In addition to primary endosymbionts, aphids may also harbour secondary endosymbionts (Degnan *et al*., 2010; Leroy *et al*., 2011; De Clerk *et al*., 2015). Secondary endosymbionts play different essential roles of protecting their aphid host, but are not involved in the aphid's survival and reproduction (Gomez-Valero *et al*., 2004; Simon *et al*., 2011; Pena *et al*., 2014; Tsuchida *et al*., 2014). The secondary endosymbionts are also not very common among aphids, even the individuals of the same species (Gil *et al*., 2004; Oliver *et al*., 2010).

Small grain crops play a crucial role as staple foods in South Africa, with maize being the most important crop followed by wheat (Department of Agriculture, Forestry and Fisheries, 2016). The past years have seen a fluctuation in wheat production (Esterhuizen and Torry, 2015; Department of

Agriculture, Forestry and Fisheries, 2016). This is due to a number of factors, which include abiotic and biotic stresses that these crops are faced with in the field, including being invaded by insects such as aphids (Atkinson and Urwin, 2012). Aphids have been shown to live in symbiotic associations with microorganisms. This association enables aphids such as the bird cherry-oat aphids to feed on crops while possibly transmitting harmful viruses. However, few efforts have been exercised in studying the endosymbiotic microorganisms of these insects in order to have a better understanding on their behaviour. This study explored the association between bird cherry-oat aphids and their endosymbiotic bacteria.

1.1 Scope of the study

Cereal crops are a major source of energy and play a vital role in nutrition and food security. However, cereal crop fields provide an ideal habitat for insects, such as aphids whose population levels normally increase to a point that can cause economic loss. Aphids have become the number one pest of small grains, causing crop damage directly through feeding on plant sap and indirectly by transmitting plant viruses. At the time of this study there were no published reports have been done on bird cherry-oat aphids and their endosymbiotic bacteria in South Africa. Studying these insects and their endosymbionts can provide valuable information on their behaviour and population growth in cereal crops. This could serve as a basis in developing effective control strategies and making improvements in the management programmes.

1.1.1 Hypothesis:

- The success of the bird cherry-oat aphid in rapid reproduction is mainly due to its symbiotic associations with endosymbiotic bacteria

1.1.2 Aim:

- To identify endosymbiotic bacteria of the South African bird cherry-oat aphid;
- To evaluate the effects of two antibiotics on bird cherry-oat aphid;
- To determine the potential for these aphids to acquire facultative endosymbionts under simulated conditions.

1.1.3 Objectives:

- To identify bird cherry-oat aphid's endosymbiotic bacteria using molecular techniques;
- To develop an approach to rear bird cherry-oat aphids under laboratory conditions;
- To assess the effect of antibiotics on the bird cherry-oat aphid using Rifampicin and broad-spectrum antibiotics;
- To examine if the bird cherry-oat aphid is able to attain secondary endosymbionts from the rose grain aphid through a shared host plant.

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Aphids are one of the most destructive pests on cultivated crops worldwide (Chapin *et al*., 2001; Tagu *et al*., 2008; Leroy *et al*., 2011; Vereschagina and Gandrabur, 2014). They are small, soft-bodied insects that come in two forms, wingless (apterae) and winged (alatae) (Ashford *et al*., 2000; Sabater *et al*., 2000; Breandle *et al*., 2006; Meresman *et al*., 2014). Most of the time they are wingless and have limited or no movements involved, their life consisting mainly of feeding and reproducing (Mira and Moran 2002; Alyokhin and Sewell, 2003). There are, however, four stimuli that cause them to move (Zhang, 2002). The first two stimuli are in response to threats by their natural enemies and poor quality of the host plant, which stimulate aphid movement in search of a new host (Muller *et al*., 2001; Parry, 2013; Meresman *et al*., 2014). The other stimuli are environmental changes, signalling that it is time to move to either primary or secondary host, and overpopulation, which stimulates aphid's migration from their present location (Fisher, 2000; Muller *et al*., 2001; Alyokhin and Sewell, 2003). Both the latter stimuli result in the production of winged forms, which assist aphids to either migrate to new plants or new areas where fresh hosts can be found (Fisher, 2000).

The high success of aphids is predominantly due to their passive migration, reproductive capabilities and feeding abilities (Fisher 2000; Morgan, 2000; Sandstrom, 2000; Tagu *et al*., 2008; Roth, 2016). Aphids feed on their host plants using specialised sucking mouthparts, a flexible tube-like structure called a stylet (Moran and Baumann, 2000; Muller *et al*., 2001; Martina 2005). The stylet, which has a food canal and a saliva canal, is inserted into the plant's phloem where the aphids feed from the plant (Blackman and Eastop, 2000; Taheri *et al*., 2010; Alkhedir *et al*., 2013; Vereschagina and Gandrabur, 2014). During this period, aphids secrete two forms of saliva, which assist the aphid in puncturing the plant and overpowering the plant's immune system as it feeds (Martina, 2005; Halarewics and Gabrys, 2012; Chaudhary *et al*., 2014; Fan *et al*., 2015; Duan *et al*., 2016).

There are over 4 000 known aphid species, 250 of which are serious pests of agricultural crops and six of which infest cereal crops throughout the world (Dedryver *et al*., 2010; Kamran *et al*., 2013). The six are *Diuraphis noxia* (RWA), *Metopolophium dirhodum* (rose grain aphid), *Rhopalosiphum maidis* (corn leaf aphid), *R. padi* (bird cherry-oat aphid), *Schizaphis graminum* (greenbug aphid) and *Sitobion avenae* (English grain aphid). Many of these aphids are monophagous (eat one kind of food) while others feed on numerous plant species (Mira and Moran, 2002). The damage these insects

inflect on plants, particularly commercial crops, has made them the worst enemies of farmers (Ashford *et al*., 2000; Fisher, 2000; Halarewics and Gabrys 2012).

One of the most significant species of aphids that is of economic importance, feeding on all major cereal crops, is *Rhopalosiphum padi* (Linnaeus), commonly known as the bird cherry-oat aphid (Jimenez-Martinez *et al*., 2004; Duan *et al*., 2016). In addition to causing serious damage to cereal crops when occurring in high numbers, bird cherry-oat aphids also plays a major role in infecting plants with harmful viruses (Jimenez-Martinez *et al*., 2004; Bosque-Perez and Eigenbrode, 2011).

2.2 Bird cherry-oat aphid description

Bird cherry-oat aphids (Figure 2.1) are a host alternating with their primary host being bird cherry (*Prunus padus* L.) and adopting cereal crops, including barley, maize, oats, rice and wheat, and other grasses as their secondary host (Glinwood and Petterson, 2000; Sandstrom 2000; Halarewics and Gabrys, 2012). In addition to alternating hosts, bird cherry-oat aphids have demonstrated a capacity to adapt to high temperatures, which has been thought to be effective in reducing populations of other aphid species (Jimenez-Martinez *et al*., 2004; Schroder *et al*., 2014). They have a behavioural mechanism for countering extreme temperature by descending to feed on the lowest parts of the plant stalk, at or below ground level (Morgan, 2000; Dunn *et al*., 2007; Michaud, 2008; Taheri et al., 2010).


a


b


c

Figure 2.1: The different forms of the bird cherry-oat aphid: a-the greenish-black wingless adult form and nymphs, b-light green wingless form and c-the winged adult form (Souce: Glinwood and Petterson 2002)

Bird cherry-oat aphids vary in colour depending on the ambient temperature and growth stage (Blackman and Eastop, 2000; Dunn *et al*., 2007; Michaud, 2008). Aphids reared under cool conditions are greenish-black and those reared under warmer conditions are light green (Figure 2.1a and 2.1b) (Dunn *et al*., 2007; Michaud, 2008; Duan *et al*., 2016). The wingless forms range from 1.2-2.4 mm, are broadly oval or pear shaped, greenish or olive brown. The head and prothorax are yellowish-brown, the legs are green and have a distinguishing characteristic of a rusty coloured pattern around the bases of their siphuncles/cornicles which are green with dusky tips (Figure 2.1b and 2.2) (Messina *et al*., 2002; Alyokhin and Sewell, 2003; Fereres and Raccan, 2015).

Winged forms are produced under unfavourable conditions, such as during a dramatic change in temperature, overcrowding and reduction in food quality (Blackman and Eastop, 2000; Muller *et al*., 2001; Breandle *et al*., 2006). Wings allow them to migrate over longer distances in search of more favourable host plants (Fan *et al*., 2015). They range from 1.8-2.0 mm in length, their body is dark green and black (Figure 2.1c), their appendages are dusky with tips of black segments and may also have the rusty brown patch on the base of their siphunicles (Figure 2.2) (Martina, 2005; Breandle *et al*., 2006; Fan *et al*., 2015).

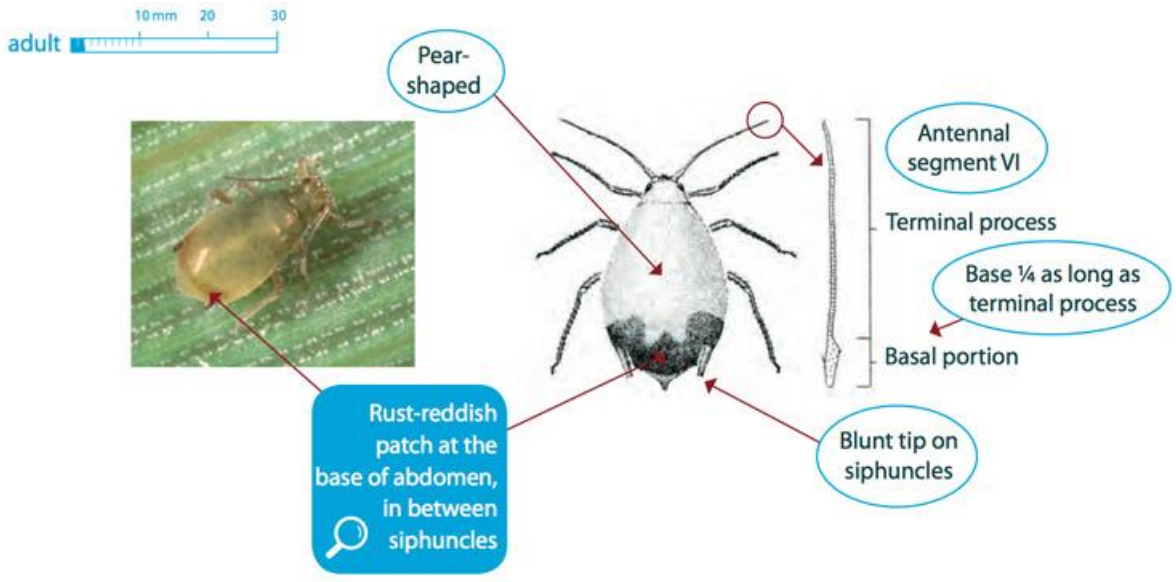

Figure 2.2: Distinctive characteristics that make-up the bird cherry-oat aphid (Source: Bellati *et a*l., 2012)

2.2.1 The life cycle of bird cherry-oat aphid

Bird cherry-oat aphids alternate between their primary winter hosts and secondary hosts (Blackman and Eastop, 2000; Morgan, 2000; Chapin *et al*., 2001; Schroder *et al*., 2014). However, under specific circumstances, such as in areas where there are no primary hosts and/or winters are warm like in South Africa, bird cherry-oat aphids are completely parthenogenetic (reproduce without fertilisation) and overwinter as adults on their secondary hosts (Dunn *et al*., 2007; Williams and Dixon, 2007; Gilabert *et al*., 2009). In such areas, the majority of individuals are females; males are rarely seen and do not contribute to the life cycle (Valenzuela et al., 2005).

Continual asexual reproduction occurs with winged female forms colonising cereal plants when available in autumn and winter, and then moving to other hosts in spring and summer (Delmotte, 2001; Gilabert *et al*., 2009). During the asexual reproduction phase, all individuals are females and they give birth to live young, which are also females. Within 1-2 days, depending on the temperature nymphs will have passed through the moulting stage and become capable of reproduction (Glinwood and Petterson, 2000; Kamran *et al*., 2013). This rapid development allows the bird cherry-oat aphids to reach tremendous population densities in a short time, their longevity being between 18-20 days per individual (Michaud, 2008).

2.2.2 Feeding mode

Bird cherry-oat aphid infestations may occur throughout plant development, from seedling to tillering stages (Dunn *et al*., 2007). They feed on plant sap by piercing the leaf, leaf stem or stem tissue, near or just below the soil line (where they are easily overlooked), depriving the plant of nutrients and therefore reducing quality (Figure 2.3) (Messina *et al*., 2002; Taheri *et al*., 2010). The aphid secretes jellifying saliva as the stylet penetrates the epidermis facilitating stylet penetration of the plant tissue by forming a hard protective sheath around the stylet as it pushes into the plant tissues (Michaud, 2008; Halarewics and Gabrys, 2012; Chaudhary *et al*., 2014). Watery saliva, which contains secondary metabolite suppressants (effectors) which suppress the plant's defence mechanism, is released as the stylet pushes through the plant's cells (Figure 2.3) (Halarewics and Gabrys, 2012; Chaudhary *et al*., 2014; Machado-Assefh *et al*., 2015; Mehrabi, 2016). The watery saliva is released until the stylet reaches the plant's phloem where it can feed on the sap for several hours to days (Halarewics and Gabrys, 2012; Mehrabi, 2016). This causes the plant's nutrients to

flow in the aphid-infested tissues, which causes a disruption in the distribution of nutrients within the plant to be reduced (Mehrabi, 2016).

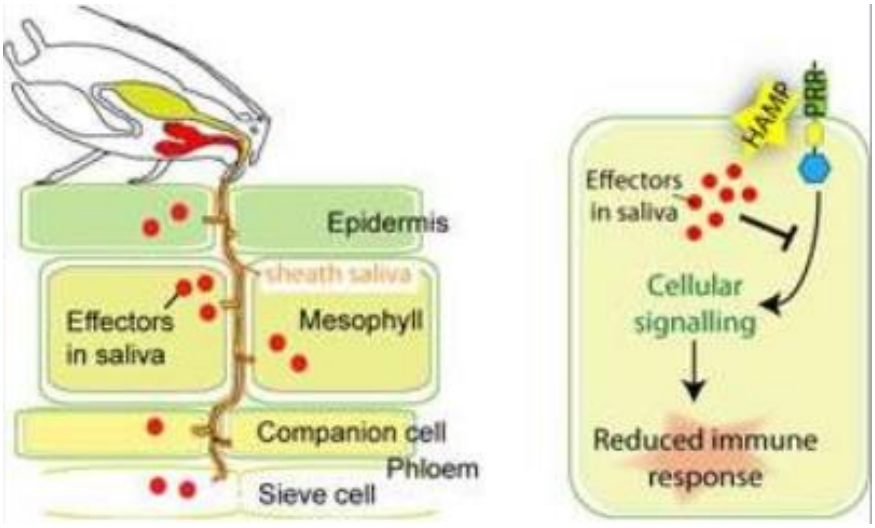

Figure 2.3: Feeding phase of aphids on a host plant showing the path of the stylet within the plant tissues releasing effectors which reduce the plant's immune system (Source: Bos and Hogenhout 2011)

2.2.3 Rearing aphids

There are a number of methods for rearing aphids that have resulted in studying these insects behaviour at a closer range. The most common is rearing of aphids feeding on host plant seedlings. The plants are usually grown in potting soil and kept in cages in greenhouses, providing environmental conditions similar to those found in the field (Gorham, 1997; Gavkare and Gupta, 2013). This method has made it possible to rear, maintain and mass produce aphid cultures. However, this method of rearing aphids requires a lot of space. Some physiological studies have made use of artificial diets (Wille and Hartman, 2008; Balvasi *et al*., 2009; Zhang *et al*., 2015). This method uses vessels, such as Petri dishes, to hold both the aphids and diet, requiring much smaller spaces. The diets are chemically defined solutions that contain amino acids, minerals, sugars and vitamins at different quantities based on the aphid species being reared (Balvasi *et al*., 2009). However, aphids reared on artificial diets are smaller in body size and have lower growth and reproduction rates (Li and Akimoto, 2018).

2.3 Aphid's endosymbiotic bacteria

Endosymbionts can either be primary or secondary, belonging to different bacterial classes within the bacterial kingdom (Table 2.1). Almost all aphid species have been shown to house the primary obligate mutualistic endosymbiotic bacteria *Buchnera aphidicola* (Buchnera, 1965; Darby *et al*., 2005; Klasson, 2005; Moran *et al*., 2005; Brinza *et al*., 2009). *B*. *aphidicola* are oval or round and 3 µm in size (Chen *et al*., 2010). Based on their genome content and similarities of orthologous genes, *Buchnera* was found to be closely related to enteric bacteria, including *Escherichia coli* (Tamas *et al*., 2001; Moran *et al*., 2005). Tamas *et al*. (2001) found orthologous pairs in the *Buchnera* genome of *Acyrthosiphon pisum* and *E. coli* genome to have an average of 62% and 89% in amino acid and 16S rDNA similarities, respectively.

The *Buchnera*'s long-term association with the aphids and the specialised functions they fulfil have influenced the rates and patterns of *Buchnera* DNA evolution (Hanses and Moran, 2011). They have extremely small genomes which range from 450-650 Kbp in length and contain 450-580 protein coding genes (Mira and Moran, 2002; Klasson, 2005; Moran *et al*., 2005). *Buchnera aphidicola* lack genes for many extracellular structures such as the genes to produce lipopolysaccharides for its outer membrane, which has resulted in them being non-pathogenic (Sabater *et al*., 2000). They have also lost genes for metabolic pathways that are involved in nutrient synthesis through deletions in their chromosomes. This has been thought to be due to their symbiotic relationship with aphids, as they share key nutrients (Wilkinson and Douglas, 2001; Wilson *et al*., 2010). The extreme reduction in their genome size is believed to have occurred during their transition from free-living to a symbiotic lifestyle (Shigenobu *et al*., 2000; Moran *et al*., 2005). Once *Buchnera* became intracellular, many genes that were previously required for survival and adaptation became unnecessary in their new environment (Mira and Moran, 2002). As a result, large parts of the genome were deleted without any disastrous outcomes (van Ham *et al*., 2002; Klasson *et al*., 2005).

In addition to primary endosymbionts, many aphids harbour a diversity of accessory bacteria known as secondary or facultative endosymbionts (Fukatsu *et al*., 2000; Oliver *et al*., 2006; Chen *et al*., 2010; Degnan *et al*., 2010). These endosymbionts are not universal across aphids, even aphids of the same species (Fukatsu *et al*., 2000; Gil *et al*., 2004). The occurrence of secondary endosymbionts varies according to temporal gradients, host plant associations and/or the presence of the aphid natural enemies (Gou *et al*., 2017). There are eight different types of secondary endosymbionts that

have been identified, with the most common ones being *Regiella insecticola*, *Hamiltonella defensa* and *Serratia symbiotica* (Gil *et al*., 2004; Oliver et al., 2006; Guay *et al*., 2009; Degnan *et al*., 2010; Henry *et al*., 2013). Secondary endosymbionts are pleomorphic (Guo *et al*., 2017), that is they have the ability to alter the shape or size in response to environmental conditions (Josh and Toleti, 2009). For instance, *S*. *symbiotica* in *Acyrthosiphon pisum* collected in USA were found to be are rod shaped whereas they were found to be large and round in *Cinara cedri* collected in Poland (Burke *et al*., 2009).

Table 2.1: Endosymbiotic bacteria found in aphids (Moran and Baumann, 2000; Gil *et al*., 2004; Oliver *et al*., 2010; Simon *et al*., 2011)

Endosymbiont	Bacterial class	Endosymbiont Classification
Buchnera aphidicola	γ-Proteobacteria	Primary endosymbiont
Hamiltonella defensa	γ-Proteobacteria	Secondary Endosymbiont
Rickettsia	α-Proteobacteria	Secondary Endosymbiont
Rickettsiella	γ-Proteobacteria	Secondary Endosymbiont
Regiella insecticola	γ-Proteobacteria	Secondary Endosymbiont
Serratia symbiotica	γ-Proteobacteria	Secondary Endosymbiont
Spiroplasma	Mollicutes	Secondary Endosymbiont
X-Type	γ-Proteobacteria	Secondary Endosymbiont
Wolbachia	α-Proteobacteria	Secondary Endosymbiont

γ-Gamma, α-Alpha

2.3.1 Location of endosymbionts within the aphid

Buchnera aphidicola are housed within large polyploid cells in the haemocoel called bacteriocytes, which are grouped into bilobed organ-like structures called bacteriome. The bacteriomes are located in the midgut or hindgut of the aphid adjacent to the ovarioles (Moran and Baumann, 2000; Breandle *et al*., 2006; Wilson *et al*., 2010). A bacteriome consists of 60-90 uninucleate bacteriocytes located in the cytoplasm, surrounded by a host derived membrane known as the symbiosome (Mira

and Moran, 2002; Brinza *et al*., 2009). In embryos, a thin layer of syncytial cells surrounds the bacteriome, whereas in adult aphids the bacteriome structure degenerates in parallel with the nutritional and reproductive decay of the insect (Wilkinson and Douglas, 2001; Brinza *et al*., 2009).

The secondary symbionts not only differ in morphology but also in their location in different lineages. Secondary endosymbionts can be found in different locations within the aphid host (Tamas *et al*., 2001; Oliver *et al*., 2006; Simon *et al*., 2011). They are located in secondary bacteriocytes, sheath cells and the haemolymph; the two latter mentioned tissues also facilitate the horizontal transfer of these endosymbionts to the next generation (Fukatsu *et al*., 2000; Gehrer and Vorburger 2012; Guo *et al*., 2017).

2.3.2 Identification of bacterial endosymbionts

The unique environment in which *B*. *aphidicola* thrives under inside its aphid host and the intimate mutualism between the two has resulted in *B*. *aphidicola* being unable to survive outside its aphid host (Fukatsu *et al*., 2000). Therefore attempts to culture this endosymbiont in axenic media under laboratory conditions have been fruitless. Attempts for culturing secondary endosymbionts have also proven difficult, but are still on-going. Only three secondary endosymbionts (*H*. *defensa*, *R*. *insecticola* and *S*. *symbiotica*) of the eight known to be associated with aphids have been cultured (Darby *et al*., 2005; Sabri *et al*., 2011; Brandt *et al*., 2017). Two of these endosymbionts (*H*. *defensa* and *S*. *symbiotica*) have successfully been cultured in cell-free media (Masson *et al*., 2018).

Darby *et al*. (2005) cultured two types of secondary endosymbionts, T type and U type, using insect cell lines. They found U type infections to be persistent, whereas T type infections were either lost when cultured for longer periods or eliminated when they were in coinfections with U type endosymbionts. Sabri *et al*. (2011) were the first to successfully isolate and culture *S*. *symbiotica* in cell-free medium containing glucose, casein peptone and yeast extract. In 2017, Brandt *et al*. cultured four *H*. *defensa* strains in TC100 medium containing 10% fetal bovine serum. These studies have opened doors that will allow biochemical profiles for these endosymbionts to be generated.

As the attempts for culturing endosymbionts in laboratory media continue, molecular techniques and microscopy are the tools being actively used to detect endosymbionts (Amann *et al*., 1995;

Fukatsu *et al*., 2000; Augustine *et al*., 2011; Rania *et al*., 2015). Microscopy has allowed morphological classification of the endosymbionts through viewing of the characteristic cell forms that occur during developmental stages (Augustin *et al*., 2011; Rania *et al*., 2015). Molecular techniques have made it possible to detect and identify unculturable endosymbiotic bacteria (Fukatsu *et al*., 1998; Dillon and Dillon, 2004; Moran *et al*., 2005; Rania *et al*., 2015). A number of these techniques are now in place to classify endosymbiotic bacteria. These include PCR-assisted sequencing of different target genes, such as 16S rDNA and GroEL genes, directly from infected aphids which allows for phylogenetic classification (Fukatsu *et al*., 2000; Rania *et al*., 2015). The PCR technique is the most commonly used for detecting and identifying aphid endosymbionts (Augustin *et al*., 2011). The in-situ hybridisation is another technique for detecting and identifying endosymbionts by characterising endosymbionts through the use of species-specific probes to directly identify bacteria under a microscope (Fukatsu *et al*., 1998; Dillon and Dillon, 2004; Moran *et al*., 2005).

2.3.3 Transmission of endosymbionts between the aphid species

Buchnera aphidicola's symbiotic relationship with aphids has been estimated to have established between 150 and 250 million years ago, when a *Buchnera* ancestor infected an aphid ancestor and has since been transmitted between aphids maternally (van Ham *et al*., 2002). Transmission of *B. aphidicola* from mother to offspring takes place during reproduction, when the host generation undergoes an infection phase (Mira and Moran, 2002; Brinza *et al*., 2009). The infection of the embryos with bacteria from mother occurs during the blastoderm stage via the opening in the posterior pole of the embryo in the viviparous morphs (Figure 2.4), whereas in the ovoviviparous morphs the eggs are the ones that get contaminated (Wilkinson *et al*., 2003; Gomez-Valero *et al*., 2004; Renoz *et al*., 2015). During the infection phase, the symbiont population passes through a successive transmission 'bottleneck'; these are transmission processes which impose severe restrictions on the number of symbionts that can gain entry into the egg or embryo (Mira and Moran, 2002; Brinza *et al*., 2009).

In 2003, Wilkinson and colleagues found that the embryo derives its bacteria from a single bacteriocyte and then undergoes rapid multiplication immediately following transmission. The embryonic *B. aphidicola* population corresponds to about 75% of the total *B. aphidicola* population of the mother (Mira and Moran, 2002). The growth rate of *B. aphidicola* reaches its peak during

embryo development, just after the young embryos have been colonised, whereas the number of *B. aphidicola* remains stable in the adult and declines as the aphid gets older (Brinza *et al*., 2009; Renoz *et al*., 2015).

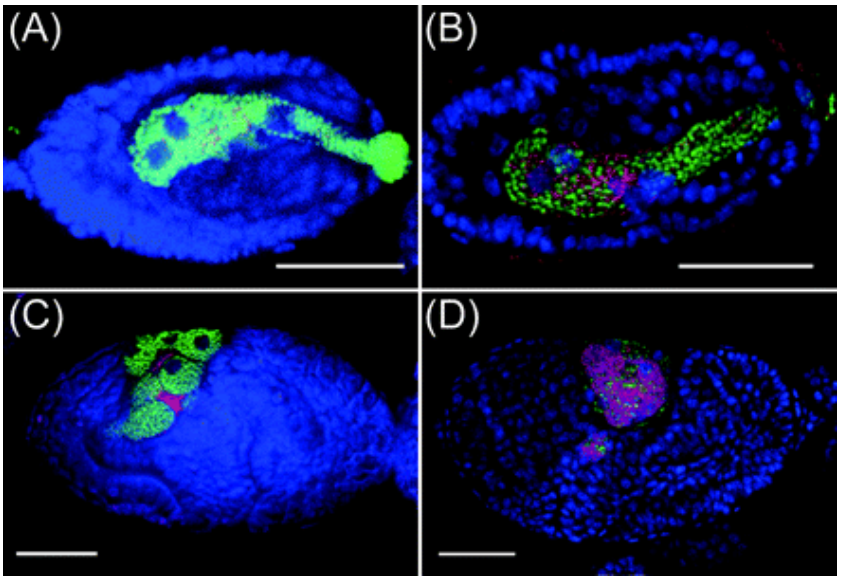

Figure 2.4: Vertical transmission of bacterial endosymbionts: green represents the *Buchnera aphidicola* infections, pink is *Serratia symbiotica* infections and blue is the host nuclei. A and B are stage 8 embryo infection, C and D are stage 12 of embryo infection (Source: Wilkinson *et al*., 2007)

Secondary endosymbionts are mainly transferred between generations via vertical transmission (Russell and Moran 2005; Oliver *et al*., 2006; Simon *et al*., 2011; Renoz *et al*., 2015). This vertical transmission route determines the prevalence of these endosymbionts, since their occurrence depends on the host reproduction. Therefore, the role the endosymbiont play on the host fitness determines its transmission between aphid's generations (Russell and Moran 2005). If the presence of the endosymbionts negatively affects the host, this might result in the loss of the endosymbiont by the aphid over a period of time (Oliver *et al*., 2006; Simon *et al*., 2011).

Secondary endosymbionts can also be horizontally transmitted on rare occasions (Oliver *et al*., 2010; Henry *et al*., 2013; Dykstra *et al*., 2014; Heyworth and Ferrari, 2015; Renoz *et al*., 2015). Horizontal transfer may be facilitated by a number of events. The aphid's natural enemies may become vectors of secondary endosymbionts by stabbing of infected aphids and then passing the endosymbiont to an uninfected aphid (Gehre and Vorburger, 2012). Infected aphids might also pass secondary

endosymbionts to a host plant, which in turn might act as a vehicle to pass secondary endosymbionts to uninfected aphids (Guo *et al*., 2017).

2.3.4 The symbiotic relationship between endosymbionts and aphids

The phloem-sap diet upon which the aphids live on is rich in carbohydrates and non-essential amino acids (Moran *et al*., 2005; Degnan *et al*., 2010; Renoz *et al*., 2015). This diet is, however, poor in essential amino acids, which are required by the aphids for their survival (Degnan *et al*., 2010; Shigenobu and Wilson, 2011; Michalik *et al*., 2014). The essential amino acids required by the aphid include, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Shigenobu and Wilson, 2011). Like other eukaryotes, aphids are unable to synthesize these amino acids and rely on *B. aphidicola* for synthesis of these amino acids (Shigenobu *et al*., 2000; Mehrabi, 2016). Despite the large-scale reduction, *B*. *aphidicola* has been shown to have retained 45-55 genes (compromising about 10% of the genome) required for the synthesis of these essential amino acids mentioned (Wilkinson and Douglas, 2001; Alkhedir *et al*., 2013; Degnan *et al*., 2010; Hanses and Moran, 2011).

The reduction of the *B. aphidicola* genome resulted in the loss of many essential genes required for its survival (Moran *et al*., 2005; Wilson *et al*., 2010). Aphids provide a unique environment for their endosymbiotic bacteria, which offers them nutrients, protection and transmission between generations (Wilkinson and Douglas, 2001; Gomez-Valero *et al*., 2004). This association has resulted in both aphids and *B. aphidicola* being entirely dependent on each other (Gomez-Valero *et al*., 2004; Renoz *et al*., 2015). The outcome of this is shown by the inability of aphids to survive and reproduce in the absence of *B. aphidicola* and by the inability of *B. aphidicola* to be isolated and cultured outside the aphid's body (Machado-Assefh *et al*., 2015). This is illustrated by an array of methods that are now in place to assess nutritional function, quantify the population and eliminate *B*. *aphidicola* from the symbiosis (Shigenobu *et al*., 2000; van Ham, 2002; Wilkinson *et al*., 2007; Machado-Assefh *et al*., 2016).

Secondary endosymbionts are not involved in the survival and reproduction processes of the host (Simon *et al*., 2011). They have been demonstrated to have diverse roles including enhancing the host resistance to natural enemies, thermal tolerance and facilitating the host in occupying new ecological niches (Gomez-Valero *et al*., 2004; Leroy *et al*., 2011; Lusasik *et al*., 2013; Pena *et al*,

2014). They can be either beneficial or detrimental to the host, depending on the environment occupied by their aphid host (Oliver *et al*., 2006; Ferrari and Vavre, 2011). In the absence of environmental challenges, carrying these endosymbionts tends to be costly for the host fitness and this may determine the frequency of secondary endosymbionts infections (Lusasik *et al*., 2013; Gou *et al*., 2017). *Hamiltonella defensa* has been shown to have a positive effect on reproduction and longevity in aphids carrying this strain in the absence of enemies (Henry *et al*., 2013). The fitness challenges arise when the host's limited resources are directed to unutilised defences, instead of the host processes that enhance growth and reproduction (Polin *et al*., 2014).

2.3.5 How endosymbionts facilitate bird cherry-oat aphids in transmitting plant viruses

In addition to aiding aphids with biosynthesis of essential nutrients that are lacking in their diet, *B. aphidicola* also increases the ability of aphids to transmit plant viruses (Goncalves *et al*., 2005; Jimenez-Martinez *et al*., 2004). B. *aphidicola* produces symbionin, a housekeeping protein that has been hypothesised to assist in folding virus particles by affining to the viral coat, giving the virus its form and stability thus protecting the virus from degradation (Cheng *et al*., 2003; Goncalves *et al*., 2005; Nagy *et al*., 2006; Medina-Ortega *et al*., 2009). This protein makes it possible for the virus particles to survive within the aphid and be able to infect other plants as the aphid continues to feed (Chapin *et al*., 2001; Medina-Ortega *et al*., 2009; Kliot and Ghanim, 2013).

2.4 Plant viruses transmitted by the bird cherry-oat aphid

Bird cherry-oat aphids play a major role in transmitting one of the most detrimental viruses of cereal grains, barley yellow dwarf viruses (BYDV) and cereal yellow dwarf virus (CYDV), which are members of the family *Luteoviridae* (Jimenez-Martinez *et al*., 2004; Gray *et al*., 2005; Ingwell *et al*., 2012). First discovered in 1951 by Oswald and Houston, BYDV are phloem limited viruses of cereal crops and other grasses (Oswald and Houston, 1951; Deb and Anderson, 2002; Goncalves *et al*., 2005; Nagy *et al*., 2006; Deb and Anderson, 2002; Fereres and Raccan, 2015). They are transmitted in a non-progressive, persistent and circulative manner, resulting in substantial grain yield losses (Li *et al*., 2001; Balaji *et al*., 2003; Goncalves *et al*., 2005; Nagy *et al*., 2006).

Since its discovery, BYDV has been classified into six strains, which are named after the aphids that transmit them. Bird cherry-oat aphids transmit three out of the six strains, namely, BYDV-*Padi*

Avenae Virus (PAV), BYDV-*Graminum Padi Virus* (GPV) and CYDV-*Rhopalosiphum Padi Virus* (Rochow, 1969). BYDV and CYDV have been reported to cause plant diseases in over 50 countries. In almost all the cases where these viruses have been identified, major losses have been due to BYDV-PAV (Wang and Abbott, 2008). Important economic crop hosts include wheat, oats, barley and occasionally rice and maize (Hawkes and Jones, 2005; Malstrom and Shu, 2005; Kumari *et al*., 2006).

2.4.1 Incidence and distribution of Barley Yellow Dwarf Virus

Barley yellow dwarf viruses are not seed-borne and cannot be transmitted mechanically (Hawkes and Jones, 2005). The virus depends entirely on the aphid for transmission (Ingwell *et al*., 2012). BYDV-infected plants emit higher concentrations of volatile organic compounds compared to those released by healthy plants (Jimenez-Ortega *et al*., 2004). As a result, aphids become more attracted to infected plants compared to healthy plants (Jimenez-Martinez *et al*., 2004; Medina-Ortega *et al*., 2009), and can acquire the virus from these plants within 30 minutes of initial feeding (Wasik and Turner, 2013).

Inside the aphid, the virus goes through a process called the latent period which usually takes between 12 and 24 h (Kliot and Ghanim, 2013; Wasik and Turner, 2013; Nega, 2014). During this period, the virus particles are transported from the foregut, passing the midgut to the haemolymph in the hindgut and are then passed on to the accessory salivary glands (Li *et al*., 2001; Kumari *et al*., 2006). The virus is then excreted through salivary ducts into the salivary canal within the stylet where it is injected into the plant's phloem through the saliva (D'Arcy and Domier, 2005; Wasik and Turner, 2013). This is known as circulative or persistent transmission because the virus is retained and circulates within the aphid's body and cannot be transmitted to the plant before this phase is completed (Krueger *et al*., 2013; Wasik and Turner, 2013; Pinherio *et al*., 2015). The virus is able to survive such a hostile environment through binding to the symbionin in the haemolymph produced by *B*. *aphidicola* (Chapin *et al*., 2001; Medina-Ortega *et al*., 2009; Kliot and Ghanim, 2013). Once they have acquired the virus, the aphid becomes a vector for life (Ingwell *et al*., 2012). The virus then spreads to other plants as the aphid moves and feeds for the rest of its lifespan (D'Arcy and Domier, 2005).

2.4.2 Disease cycle

The virus is deposited into the plant's phloem with the watery saliva as the aphid feeds (Figure 2.5) (D'Arcy and Domier, 2005). Once inside the plant, the virus nucleic acid (+ssRNA) is released from the coat protein and gets amplified using the host's translational machinery (den Boon *et al*., 2010; Nega, 2014). During the amplification process, many complementary copies (-ssRNA) of the released viral +ssRNA are produced (Novoa *et al*., 2005). The new copies then make more copies of the virus, which get assembled into subgenomic nucleic acid and structural proteins to form new virus particles (D'Arcy and Domier, 2005). These new virus particles then get inserted into the new cells of the same plant. The virus particles can also be ingested by aphid vectors and be transported to other parts of the same plant or a new plant, where the process can resume again (Brault *et al*., 2006; Peter *et al*., 2009; Fereres and Raccan, 2015).

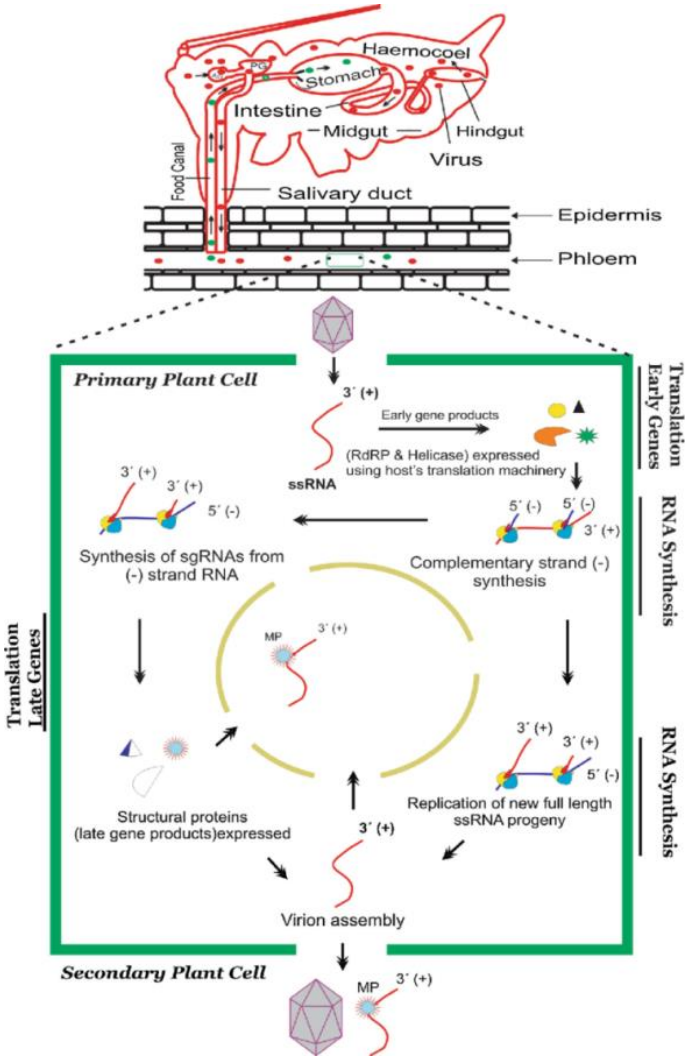

Figure 2.5: Replication cycle of Barley yellow dwarf virus within a plant cell (Ali *et al*., 2014)

2.5 Symptoms resulting from the bird cherry-oat infestations

Bird cherry-oat aphid feeding does not significantly affect grain yield, however, heavy infestations may lead to reduced grain quality, affecting protein content and test weight (Morgan, 2000; Parry *et al*., 2013; Meresman *et al*., 2014). Aphids also produce honeydew which not only attract ants and mould, but also affects photosynthesis processes of the plant (Morgan, 2000; Parry *et al*., 2013; Meresman *et al*., 2014). Major yield losses that lead to an economic impact on the grain production result from BYDV-carrying bird cherry-oat aphids (Nagy *et al*., 2006; Nega *et al*., 2014). The infection and subsequent death of the phloem cells inhibit translocation of nutrients, slow down plant growth and induce loss of chlorophyll, resulting in characteristic symptoms (Gray and Gildow 2003; D'Arcy and Domier, 2005; Fereres and Raccan, 2015).

The genotype, age, physiological conditions of the host plant, as well as the strain of the virus and environmental conditions may affect symptoms (Ali *et al*., 2013; Meresman *et al*., 2014). Visible symptoms usually do not appear until the aphids are gone, which may lead to misdiagnosis such as environmental stress or nutritional disorders (Nagy *et al*., 2006; Parry *et al*., 2013). Symptoms may include yellow to red-purple leaf discolouration (Figure 2.6), curled leaves, winterkill, and relatively small-irregular plants (Nega, 2014; Roth, 2016). BYDV infections may lead to under-developed root system, delayed maturity, nutritional disorders and reduced grain and quality (Alyokhin and Sewell, 2003; Nega, 2014). Early infections, immediately following emergence can lead to the greatest impact on production (Messina *et al*., 2002; Taheri *et al*., 2010; Dunn *et al*., 2007).

Figure 2.6: Symptoms induced by barley yellow dwarf virus on cereal grains. The images show leaf discolouration symptoms on different cereal crops, starting from the left showing the yellowing of wheat leaves, purple discolouration of oats in the middle and yellowing of barley on the right

2.6 Management Strategies

Crop protection is essential in safe guarding agricultural production from insects (Dedryver *et al*., 2010). In order for a control strategy to work, it requires an in-depth background of the aphid. This allows for a better understanding of how the aphid grows, survives, reproduces and the rate in which its population increases (Descamp and Chopa, 2011). Studying the behaviour of aphids has allowed for such information to be used to develop chemicals, resistant cultivars and biological control agents (BCA) as potential management strategies. A resource that has not been tried on a larger scale is controlling of endosymbionts, specifically *B*. *aphidicola*, as a management strategy to control aphids. An insect that requires its endosymbiont for survival and reproduction is vulnerable to interventions that target either the endosymbiont or specific insect-endosymbiont interactions (Douglas, 2007). The purpose of such a stratergy is not to eradicate the insect but eliminate its harmful effects to levels that are not of economic losses. This stratergy thus far relies mostly on antibiotics, which have been shown to suppress insect populations (Koga *et al*., 2007; Machaddo-Assef *et al*., 2015). Antibiotics are, however, unacceptable for commercial use due to its resulting harmful effects on both the environment and humans. The challenge is to identify alternative routes to disrupt the endosymbionts.

2.6.1 Use of chemicals

The use of pest insecticides is the most common practice of managing aphids since their introduction in the 1940s (Foster *et al*., 2002; Puinean *et al*., 2010; Bhatia *et al*., 2011; Chougule and Bonning, 2012). This form of control is immediate but only decimates localized aphid populations temporarily (Stern *et al*., 1959). There are a number of effective insecticides commercially available. However, overuse has contributed to resistance among aphids to many classes of compounds including organophosphates, carbamates and pyrethroids (Sadeghi *et al*., 2009; Puinean *et al*., 2010). For instance, bird cherry-oat aphid has been shown to have developed resistance to imidacloprid systemic insecticide (Wan *et al*., 2018). The use of insecticides is also costly and poses potential threats to both humans and the environment (Bhatia *et al*., 2011).

2.6.2 Resistant cultivars

The use of resistant cultivars has demonstrated to be the most effective control measure, as it is more cost effective, environmentally appropriate and has resulted in some success in the management of some agricultural pests (Ohm and Anderson, 2007; Chougule and Bonning, 2012).

The process involves phenotyping of plant collections in search for beneficial plant traits, such as those that confer resistance, to develop new germplasm that will thrive under environmental pressures (Thomas *et al.,* 2016). Plant genes that confer resistance are introduced into cultivated varieties of crops. A number of RWA resistance genes have been identified over the years in wheat cultivars for example. In South Africa, RWA resistant genes, such as *DN1*, have been found and are used in wheat breeding programmes (Lui et al., 2001). However, sources of plant resistance to aphids are limited and regardless of its role as a significant insect of cereal crops, there have not been any developments in breeding programmes for resistance against bird cherry-oat aphids (Crespo-Herret *et al*., 2014). There are wheat cultivars that have shown to reduce the numbers of bird cherry-oat infestations, however, no genetic analyses has been made to identify the source of resistance (Girvin et al., 2017).

2.6.3 Biological control agents

Use of BCA is a natural and environmentally friendly method to reduce crop damage resulting from insect infestation (Pal *et al*., 2006). This has made BCA the main part of intensive pest management. Aphids are preyed upon by a number of parasites and predators (Bale *et al*., 2008; Boivin *et al*., 2011). These have in turn been used in strategies to control aphid infestations under controlled conditions where variability in sensitivity has been observed in different aphids (Snyder and Ives, 2003; Scarborough *et al*., 2005; van Lenteren *et al*., 2006). This variability in sensitivity might be due to the presence or absence of secondary endosymbionts. Secondary endosymbionts have been shown to protect their aphid host from parasites and predators. For instance, Scarborough *et al.* (2005) showed *R. insecticola* to protect pea aphids from *Pandora neoaphidis* (a fungal insect pathogen and an obligate pathogen to aphids). Apart from aphids having gained resistance to their natural enemies with the assistance of endosymbionts, the field environment differs greatly from the controlled conditions offered by the greenhouses. For instance, the uncontrolled temperature in the field might interfere with the positive effects of BCA in controlling aphids (Miller and Rebek, 2018).

2.6.4 Controlling endosymbionts as a means of controlling aphids

The past few decades have resulted in a massive biological exploration of aphids and their endosymbionts. These studies have made it apparent that the functions of endosymbiotic bacteria differ greatly in their aphid host. Aphids have also been shown to perform poorly in the absence of

their endosymbiotic bacteria, particularly in the absence of *B*. *aphidicola* (Griffits and Beck, 1974; Liadouze *et al*., 1994; Cheng *et al*., 2010; Machado-Assefh *et al*., 2015; Zhang *et al*., 2015). Elimination of endosymbionts has been shown to be obtained through the use of antibiotics (Davies, 1990; Chopra et and Roberts, 2001). The use of antibiotics has allowed the study of the behaviour of the aphid host without their endosymbionts. This has lead to the notion that controlling/eliminating endosymbionts could lead to aphid management, by reducing aphid survival and reproduction rate to numbers that will not result in economic loss of the crops (Douglas, 2012).

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CHAPTER 3: DETECTING THE ENDOSYMBIOTIC BACTERIA OF BIRD CHERRY-OAT APHID

3.1 Abstract

Endosymbiotic associations, where eukaryotes serve as hosts to microbial communities are abundant in nature. These endosymbionts play a significant role in the upkeep of their hosts, making some of these relationships obligatory, while some are not so predominant and can either have positive or negative effects on the host. However, endosymbiotic studies of bird cherry-oat aphid microbiota have not been explored in South Africa. This study investigated the endosymbiotic bacterial communities of the South African bird cherry-oat aphid using 16S rDNA. PCR techniques were employed, using species-specific diagnostic markers, to determine the variability of bacterial species that are harboured by these aphids. The markers used showed some variety in the bacterial populations of the aphids that were targeted for this study. The primary endosymbiotic bacterium, *B*. *aphidicola*, screened positive in all the samples. This was not surprising as aphids have an obligatory relationship with the primary endosymbionts. The opposite was observed for secondary endosymbionts, which only *H*. *defensa* screened positive in the samples screened while no amplification was observed for the other five secondary endosymbionts that were targeted in this study. This shows that secondary endosymbionts are not very common amongst this aphid species.

Keywords: *Bird cherry-oat aphid; endosymbiotic associations; microbial communities; primary endosymbionts; secondary endosymbiont; PCR detection; 16S rDNA*

3.2 Introduction

Symbiotic associations where one organism lives inside the other are an essential part of life (Gil *et al*., 2004; Kolsch and Synefiaridou, 2012; De Clerk *et al*., 2015; Gauthier *et al*., 2015). The close association between the host and the symbiont results in very close biological interactions and inter-dependency between such partners, generating novel biological properties (Padro *et al*., 2009; Mitchell, 2014). In many cases, this integration becomes an inseparable biological entity (Gomez-Valero *et al*., 2004), making it impossible to culture the endosymbiont in generic laboratory media (Ateyyat, 2008). The intracellular location of the symbiont requires that it's host supplies it with energy and ensures transmission to the next generation (Wilkinson *et al*., 2001; Gomez-Valero *et al*., 2004). While the symbiont aids its host with increasing reproduction or make it possible for their

host to colonise new habitats (Kolsch and Synefiaridou, 2012). For instance, aphids are unable to synthesise that are vital for its upkeep and they obtain these nutrients from the endosymbionts that they house (Russel and Moran, 2006).

Microorganisms are frequent participants of such associations. They are mostly bound in mutually beneficial symbiotic partnerships with the host which is mostly, if not always, a eukaryote (van Ham *et al*., 2002; Russell and Moran, 2006; Gauthier *et al*., 2015). Eukaryotes display great diversity and morphological complexity but have limited metabolic capabilities. Eukaryotes, such as aphids are unable to perform certain tasks that are essential for their well-being, including nitrogen fixation which is vital for the synthesis of essential amino acids (Sabater *et al*., 2001; Wilkinson *et al*., 2001; Zhao *et al*., 2016). Aphids are able to obtain fixed nitrogen through their symbiotic interaction with their primary endosymbiotic bacteria (Kneip *et al*., 2007), *Buchnera aphidicola*, an obligate but mutualistic endosymbiont, which is essential for their survival and reproduction (De Clerk *et al*., 2014, Zhang *et al*., 2015).

Aphids may also harbour accessory symbionts commonly known as secondary or facultative endosymbionts (Simon *et al*., 2011; Renoz *et al*., 2015). Several studies have identified seven secondary symbionts in the pea aphid, *Acyrthosiphon pisum*, five of which belong to the Gammaproteobacteria (*Hamiltonella defensa* (PABS (T)), *Regiella insecticola* (PAUS), *Rickettsiella* sp., *Serratia symbiotica* (PASS (R)), and X-type), one to the Alphaproteobacteria (*Rickettsia* sp. (PAR)) and one to Mollicutes (*Spiroplasma* sp.) (Simon *et al*., 2011; Gauthier *et al*., 2015). The most common secondary endosymbionts with clearly defined roles have been shown to be those that belong to the γ-Proteobacteria. *H*. *defensa* and *S. symbiotica* are known to protect the aphid from parasitic wasps and heat shock (Guay *et al*., 2009; Vorburger *et al*., 2010). *R. insecticola* is said to protect the aphid from pathogenic fungi (Scarborough et al., 2005). X-type protects the host from hymenoptera wasps (Lusasik *et al*., 2013) and *Rickettsiella* sp. changes the phenotypic characteristics (colour) of the host, making the host unrecognizable to it's enemies (Tsuchida *et al*., 2010).

The secondary endosymbionts make it possible for the host to survive hostile environments. The occurrence of *Rickettsia* and *Spiroplasma* sp. has been found to be rare and their role is still not clear. Studies done by Tsuchida *et al*. (2002), Russell and Moran (2006) and Simon *et al*. (2011) found these to be parasitic and reproduction manipulators, while others such as Lusasik *et al*. (2013) have

found these two to have protective effects for the host against pathogenic fungi. These studies suggest that the environment might be an essential contributor to how the endosymbionts behave within their aphid host (Guo *et al*., 2017).

The biological importance of endosymbionts to the aphids has resulted in aphid-symbiosis association being the most studied of the Insecta class (Oliver *et al*., 2010; Pena *et al*., 2014). Although endosymbiotic microorganisms of aphids have been extensively studied, there is very limited data in South Africa about the diversity of aphid microbiota. Bird cherry-oat aphid, *Rhopalosiphum padi*, is among the most serious pests of cereal crops worldwide (Wilkinson *et al*., 2003; Jimenez-Martinez *et al*., 2004; Medina-Ortega *et al*., 2009). In South Africa, bird cherry-oat aphid is considered one of the most economically important aphids of wheat causing 15% to 33% loss/damage through feeding and transmission of plant viruses in wheat (Prinsloo, 2017). In order to reach a better understanding on the behaviour of these aphids, it is important to know the microbiota that the bird cherry-oat aphids contain. Since endosymbionts cannot live outside their hosts, they have proven difficult to culture under normal laboratory conditions. However, advances in molecular biology have made it possible to identify these symbionts. This study took advantage of these technologies and the conventional polymerase chain reaction (PCR), in order to detect and identify endosymbiotic bacteria of the bird cherry-oat aphid.

3.3 Materials and methods

3.3.1 Collection and counting of aphid samples

The bird cherry-oat aphid colony was originally collected from parts of Western Cape and KwaZulu-Natal. The aphids were cultured on BSP-SNR-04-2015 wheat cultivars in 12 cm depth × 14 cm diameter pot plants in growth chambers at 22^{o}C with a photoperiod of 13 h light (13L): 11 h dark (11D) in the greenhouses at the Agricultural Research Council-Small Grain (ARC-SG) in Bethlehem, South Africa. Six samples were collected from different cubicles in the greenhouse by randomly selecting bird cherry-oat aphid infested wheat pot plants (Table 3.1). The seventh sample was collected from a bird cherry-oat aphid infested maize leaf in the field in Bethlehem, South Africa (Table 3.1). In order to determine the diversity of endosymbionts living inside the bird cherry-oat aphids, a pool of fifty healthy and wingless adult aphids were collected into a 2 ml Eppendorf tube containing 500 µl of absolute ethanol using a dissecting microscope (SMZ800N Zoom Stereomicroscope, Nikon Optiphot Japan).

Table 3.1: Bird cherry-oat aphid samples collected for detection of endosymbiotic bacteria. The samples from the greenhouses were kept in numbered cages and were therefore labelled according to the numbers of the greenhouses and the cages they were collected from

Sample	Where sample was collected
1	Cage 1 in Greenhouse 7a
2	Greenhouse 5
3	Subculture of cage 1 in Greenhose 7a
4	Subculture of cage 2 in Greenhouse 7a
5	Cage 2 in Greenhous 7a
6	Greenhouse 8a
7	Maize field in Bethlehem

3.3.2 Isolation of 16S rDNA

3.3.2.1 DNA extraction

Aphids were surface sterilised by washing twice with 70% ethanol and removing the excess ethanol by rinsing three times with sterilised distilled water. Total DNA was extracted using a modified Cetyl Trimethylammonium Bromide (CTAB) method (1 M Tris (hydroxymethyl) aminomethane Hydrocloride (Tris-HCl) pH 8.0, 0.5 M Ethylenediaminetetraacetic acid (EDTA), 2% CTAB, 5 M Sodium Chloride (NaCl) and β-mercaptoethanol) according to Perez-Lopez and Pantoja (2014). Seven hundred and fifty micro litres of pre-warmed CTAB extraction buffer and 2 stainless steel beads were added to the fifty adult aphids in a 2 ml Eppendorf tube. The aphids were homogenised using Qiagen Retsch 85210 TissueLyser (Hilden, Germany) at the 30.0 Hz for 5 min. The homogenate was then incubated at 37^{o}C for 40 min. Equal volumes of chloroform: isoamyl alcohol (24:1) were added to the homogenate and centrifuged at 10 000 x *g* for 5 min. The supernatant was transferred to a 1.5 ml Eppendorf tube containing 500 µl of 100% isopropanol and incubated at room temperature for 2 h. The tube was then centrifuged at 10 000 x *g* for 5 min, the supernatant was discarded and the pellet was washed with 70% ethanol. The tubes were centrifuged at 10 000 x *g* for 5 min. The supernatant was discarded and the pellet was left to air dry at room temperature for 1 h. The pellet was then resuspended in 100 µl 1× Tris-EDTA buffer and 1 µl RNase A (AMRESCO®, Ohio, USA) and

incubated at 37^{o}C for 1 h. The DNA concentration and quality of DNA was determined by monitoring $A_{260/280}$ and $A_{260/230}$ (Table A1) absorbance ratios using the NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, USA). The total DNA was then diluted to 50 ng/µl using 1× TE buffer.

3.3.2.2 Detecting endosymbionts of Bird cherry-oat aphid

The endosymbiotic bacteria of bird cherry-oat aphids were detected using PCR techniques. The presence of the primary endosymbionts from the samples were evaluated using the *Buchnera aphidicola* specific primer set ApisP1_nt298:5'TTCCAGTGTGGCTGGTTA3' and 16SA1_nt1:5'AGAGTTTGATCMTGGCTCAG3' (De Clerk *et al*., 2015) in end point PCR reaction. A nested PCR was carried out for the secondary endosymbiotic bacteria. Universal 16S rDNA bacterial primer pair, F: 5'GCTTAACACATGCAAG3' and R: 5'ACGGGCAGTGTGTACAAGACC3' was used in the first amplification to enrich the bacterial sequence over the aphid's genomic sequence in the total DNA template. The PCR reaction was carried out in a final volume of 15 µl, which contained 2× KAPA Taq ReadyMix with loading dye (KAPABIOSYSTEMS, Cape Town, South Africa), 0.5 µM each of the forward and reverse primers and 50 ng/µl of the DNA template. The thermal cycler (Bio-Rad: MY Cycler, California, USA) parameters were the initial denaturation step set at 95^{o}C for 5 min followed by 30 cycles of 95^{o}C for 30 s, 55^{o}C for 30 s, 72^{o}C for 30 s and final extension step at 72^{o}C for 10 min. The amplicons were run on a 1.5% agarose gel which was stained with final concentration of 1× SYBR®SafeDNA gel stain (Invitrogen, Thermo Fisher Scientific, Waltman, USA) visualised and captured on a Molecular Imager® GelDoc™ XR+ Imaging System (Biorad, California, USA).

A 100 µl final volume of the universal 16S bacterial amplicons was prepared by diluting 7 µl PCR product with 93 µl RNase free water. The species-specific primers for secondary endosymbiotic bacteria identification (Table 2) were then used for the second PCR, using the diluted amplification product from the first PCR reaction as a template. The reactions were carried out in a final volume of 15 µl, which contained 2× KAPA Taq ReadyMix with dye, 0.5 µM each of the forward and reverse primers and 4 µl of the diluted universal 16S rDNA bacterial primer PCR product as the DNA template. The thermal cycler (Bio-Rad: MY Cycler, California, USA) parameters were set as follows, the initial denaturation step at 95^{o}C for 5 min followed by 30 cycles of 95^{o}C denaturation temperature for 30 s, annealing temperature (Table 3.2) for 30 s, 72^{o}C for 30 s and final extension step at 72^{o}C for 10 min. The amplicons were run and visualised as mentioned above. The positive amplicons were then taken to Inqaba Biotech, South Africa for sequencing. The sequence was

cleaned using DNAman 6.0 and the identity of the endosymbionts was revealed by aligning the sequences to NCBI BLAST.

Table 3.2: Primers used for detecting secondary endosymbiotic bacteria of bird cherry-oat aphids, from Ferrari *et al*., 2011 and Pena *et al*., 2014

Symbiont species	Forward (F) and Reverse (R) Primer	Expected size (bp)	Annealing temperature (°C)
Hamiltonella defensa	10F:5'AGTTTGATCATGGCTCAGATT3'	490	57
	T419R:5'AAATGGTATTCGCATTTATCG3'		
Regiella insecticola	10F:5'AGTTTGATCATGGCTCAGATT3'	470	57
	U443R:5'GGTAACGTCAATCGATAAGCA3'		
Rickettsia	16SA1:5'AGAGTTTGA TCMTGGCTCAG3'	591	45
	Rick16SR:5'TTTGAAAGCAATTCCGAGGT3'		
Serratia symbiotica	10F:5'AGTTTGATCATGGCTCAGATT3'	890	57
	R443R:5'CTTCTGCGAGTA ACGTCAATG3'		
Spiroplasma	10F:5'AGTTTGATCATGGCTCAGATT3'	600	45
	TKSSsp:5'ATCATCAACCCT GCCTTT3'		
X-Type	10F:5'AGTTTGATCATGGCTCAGATT3'	450	57
	X420R:5'GCAACACTCTTTGCA TTGCT3'		

3.4 Results

In this study, the endosymbiotic bacteria of bird cherry-oat aphids were explored using molecular biology techniques. Seven samples, each collected in duplicates resulted in fourteen bird cherry-oat aphid samples being screened. Due to the lack of population and sequencing information of the aphid's microbiota in South Africa, there were no positive controls used in this study. *Escherichia coli* was used as a negative control in order to test the specificity of the species-specific primers. This resulted in a total number of 15 screened samples per primer pair, the first sample loaded on all the electrophoresis gels being *E*. *coli*.

The presence of the primary endosymbiont was screened using *B*. *aphidicola* specific primers. The results showed an amplification of the expected 321 bp band size for all seven samples (Figure 1). For the *E*. *coli* sample, (Figure 3.1 sample 1), no amplification was observed with *B*. *aphidicola* specific primers. This was taken as an indication that the primers used were specific to *B*. *aphidicola* species. To confirm the amplicons obtained were truly *B*. *aphidicola*, the samples were sent for sequencing validation. In order to reveal the identity of the primed amplicons, the sequence (Appendix (page 90)) was aligned to the NCBI BLAST database. The results obtained after sequencing confirm that the bird cherry-oat aphids collected from different cubicles in the greenhouses at the ARC-SG and a maize field in Bethlehem, all carry the obligate primary endosymbiotic bacterium, *B*. *aphidicola*.

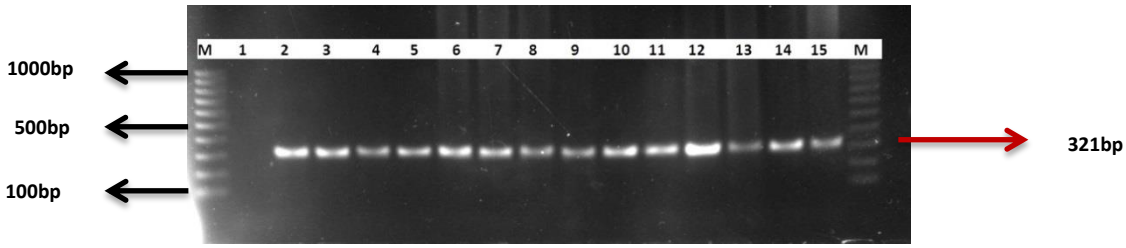

Figure 3.1: Detected primary endosymbiotic bacteria of the Bird cherry-oat aphids using *Buchnera aphidicola* species specific primers

Once the primary endosymbiont's presence was confirmed, the presence of secondary endosymbionts was then screened for in a nested PCR. The first PCR induced the DNA template of bacterial species over the aphid's DNA in the total bird cherry-oat DNA template using universal 16S rDNA bacterial primer pair. The expected band size for this primer pair was 1500 bp. A presence of bacteria was observed when the total DNA samples of bird cherry-oat aphids were amplified with the universal 16S rDNA bacterial primer. This was shown by the presence of the expected band sizes in Figure 2 for all the samples screened, which amplified at the expected 1500 bp band size. This showed that total DNA from bird cherry-oat aphids contained an expected diversity in bacterial species. The presence of the faint band when *E*. *coli* was used (Figure 3.2 sample 1) was also expected as this primer is selective for all bacterial species. This was also taken as an indication that the universal 16S rDNA bacterial primer pair had enriched the bacterial DNA over the predominant aphids genomic DNA.

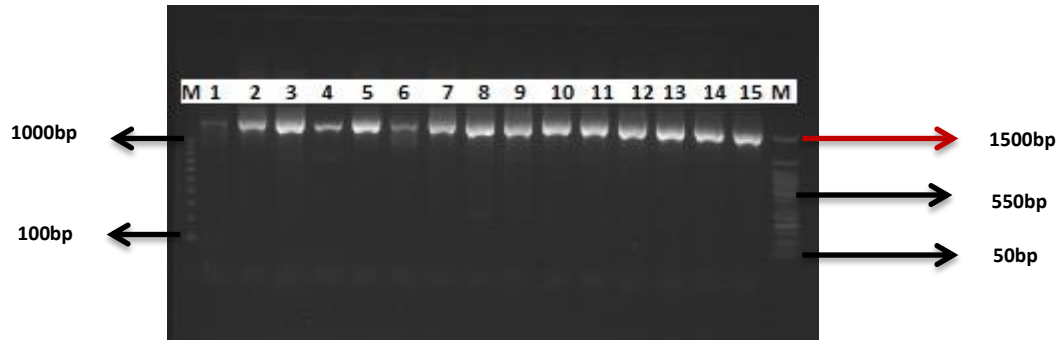

Figure 3.2: Bacterial species harboured by the Bird cherry-oat aphid detected with the universal 16S rDNA bacterial primers in a first reaction of the nested PCR

The amplicons obtained from using universal 16S rDNA bacterial primers were then diluted and used to select for secondary endosymbionts of the bird cherry-oat aphids using primers that are specific to six of the eight endosymbionts that are known to be associated with cereal aphids. The species specific primers for secondary endosymbionts showed completely different findings from that of the primary endosymbionts (Figure 3.3). A positive amplification of the expected 490 bp band, *H. defensa*, was only observed in samples 9, 10 and 11 (Figure 3.3A). The true identity of the positive 490 bp amplification was confirmed through sequencing and sequence aligning (Appendix (page 90)) on NCIB BLAST that the bacterium was indeed *H. defensa*.

The other five secondary endosymbionts, *Rickettsia* (Figure 3.3B), *R. insecticola* (Figure 3.3C), Spiroplasma (Figure 3.3D), *S. symbiotica* (Figure 3.3E) and X-type (Figure 3.3F) did not amplify in any samples. This indicated the absence of these targeted secondary endosymbionts in these samples. The only amplification observed, when primers specific to these endosymbionts were used, was the one that was observed in Figure3. 2 when universal primers were used in the first PCR. Since the PCR products of this primer were used as a template for the secondary specific primer PCR, it would be expected for them to also appear in the background.

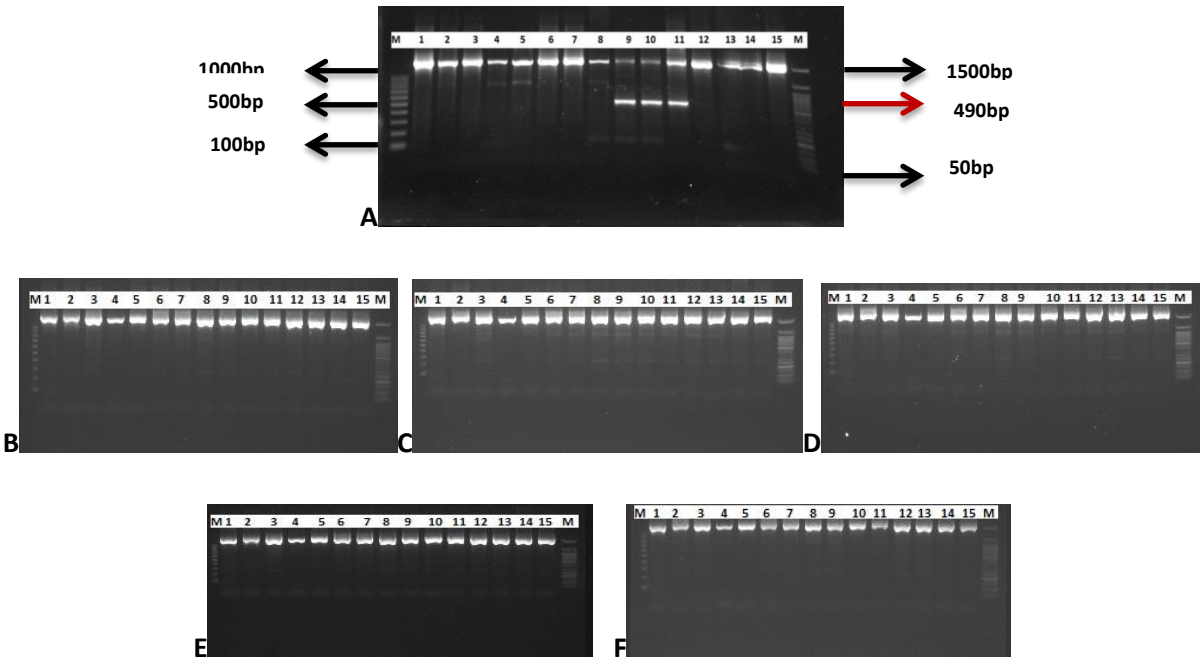

Figure 3.3: Detection of secondary endosymbiotic bacteria of the bird cherry-oat aphids using species specific primers in the second reaction of a nested PCR

3.5 Discussion

This study investigated the presence of endosymbiotic bacteria harboured by the South African bird cherry-oat aphid. The primary endosymbiont, *Buchnera aphidicola*, was detected in all the aphid samples screened. This is shown by the presence of the expected fragment size at 321 bp (Figure 3.1) for all the samples. These results confirm and support previous studies that have indicated that this endosymbiont persists in almost all the aphid species. This is an anciently acquired bacterium and the most important bacterial symbiont associated with aphids (Moran *et al*., 2005; Gauthier *et al*., 2015). *Buchnera aphidicola* is significant in the aphid's nutrition, synthesizing vital nutrients, such as the ten essential amino acids, lacking in the aphids diet (Wilson *et al*., 2010). This makes it essential for the survival and reproduction of its host (Darby *et al*., 2005; De Clerk *et al*., 2014; Zhang *et al*., 2015).

Unlike primary endosymbionts, this study found the prevalence of secondary endosymbionts to be sporadic in the screened samples. However, this is not surprising as it has been shown that some species do not harbour any secondary endosymbionts, while others carry one or more (Oliver *et al*., 2010, Pena *et al*., 2014). For instance, it has been reported that the pea aphid *Acyrthosiphon pisum*

can carry at least seven genera of secondary endosymbionts (Lusasik *et al*., 2013), whereas Pena *et al*. (2014) did not find any secondary symbionts in *Schizaphis rufula*. The present study showed a clear presence of secondary endosymbionts in sample 9, 10 and 11 (Figure 3.3A), which showed the presence of *H*. *defensa*. These samples were collected from cages kept in glasshouse 7a, sample 10 and 11 were collected from cage 2 and sample 9 was collected in the cage that was the subculture of cage 2 (Table 3.1). This shows that secondary endosymbionts from cage 2 were able to be transferred to their offspring when this colony was subcultured. It should be noted that only one of the two samples collected from subculture of cage 2 had positive amplification. Apart from the three samples that screened positive for *H*. *defensa*, the screened samples did not show the presence of any of the other five screened secondary endosymbionts. Willie and Hartman (2009) detected *B*. *aphidicola*, *H*. *defensa*, *R*. *insecticola* and *S*. *symbiotica* on Soy bean aphids. They found all the Soy bean aphid samples to contain the obligatory *B*. *aphidicola* and no secondary endosymbionts were detected, instead they found *Arsenophonus* sp., which is a symbiont that is associated with whiteflies. Pena *et al*. (2014) also conducted a study to detect secondary endosymbionts of *Laingia psammae*, *R*. *padi* and *S*. *raful*. Their findings showed a great variation of secondary endosymbionts amongst these aphids, with *S*. *raful* containing three endosymbionts, while *L*. *psammae* and *R*. *padi* only contained *S*. *serratia*. This could be an indication that the presence of secondary endosymbionts differs at population level even within the same species occupying the same locality.

Secondary endosymbionts have demonstrated to have diverse roles, which can either be positive or negative for their aphid host, that is, enhancing host resistance to natural enemies or negatively affecting the host's fitness through reducing reproduction rate (Gil *et al*., 2004; Degnan *et al*., 2010, Guo *et al*., 2017). However, these roles have been shown to be mostly positive to the host, which brings about a question; if these endosymbionts are beneficial to the aphid, why is their occurrence so sporadic? (Vorburger *et al*., 2010). It has been suggested that the environmental conditions and co-infection strongly influence the presence of secondary endosymbionts within the aphid host and the role of the endosymbiont, respectively (Russell *et al*., 2003; Guo *et al*., 2017). This is because carrying the endosymbionts tends to be costly to the host, especially if they are not beneficial to the host. Endosymbionts that do not offer any benefits to the host's fitness eventually get lost from the population as carrying them imposes a potential fitness cost to the host (Russell *et al*., 2003; Scarborough *et al*., 2005). However, it must be noted that this natural selection by the host takes years and over many generations, in the laboratory or greenhouses experiments (Oliver *et al*., 2010).

Hamiltonella defensa is known to protect its host from parasitic wasps (Russell and Moran 2006; Guay *et al*., 2017). The majority of the samples were collected from the greenhouses where they live in a protective environment in which natural enemies are less likely to occur, and are constantly provided food to prevent overcrowding. The environmental conditions are kept at a constant favourable temperature. It would have been expected that the sample collected from the maize field would harbour secondary endosymbionts as they would be more beneficial to them than the samples collected from the greenhouses. However, this was not the case in this study. *H*. *defensa* was detected in two of the six samples collected from the greenhouses and not in the sample collected in the maize field. Further investigations are required as it is not clear why the aphids are harbouring the endosymbiont under in which carrying them would be more of a cost than a benefit. It is possible that this endosymbiont might be serving a different function to the aphids. It is also possible that the aphids might be in the selection process for this endosymbiont, as they were only detected in sample 9 and not in sample 8 as these were collected in the same cage.

In comparing population dynamics of defensive symbionts, *H*. *defensa* and *S*. *symbiotica*, in *A*. *pisum*, Oliver *et al*. (2008) found a significant decline in the frequency of both *H*. *defensa* and *S*. *symbiotica* in the absence of *Aphidius evri*. They observed the opposite when *A*. *pisum* was in the presence of *A*. *evri*, both the frequency of these endosymbionts and aphid reproduction increased. They concluded that the declining frequency of *H*. *defensa*-infected aphids in the absence of parasitism indicated a probable cost to infection. They also added that despite the fitness benefits offered by these endosymbionts, they are not fixed within the aphid populations. Their presence is mostly guaranteed only in the presence of natural enemies. Pena *et al*. (2014) collected aphid population at the most extreme environmental conditions in the coastal dunes of the North Sea. The found all the aphid populations to cantain *S*. *symbiotica*, whereas *H*. *defensa* and X-type endosymbionts were found in only one sample. It is expected for all the samples to carry *S*. *symbiotica* as it has been shown to protect aphids from environmental changes. However, they could not explain the sporadic occurrence of H. defensa and X-type endosymbionts. They also suggested that further studies were required to provide insight on the functions of these endosymbionts in these aphids.

3.6 Conclusion

The findings of this study have shown that the South African bird cherry-oat aphid does harbour endosymbiotic bacteria. However, in establishing this, the study has also raised more questions, such as what does the presence of these endosymbionts mean for this aphid and how does this compare with the aphids that do not harbour these endosymbionts. In addition, how do the

endosymbionts from the aphids kept under optimum conditions differ from that of the aphids found under field conditions? Going forward, detection of endosymbionts should be paired with investigating the roles of the detected endosymbionts on such aphids in order to determine the influence of environmental conditions on the endosymbionts.

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CHAPTER 4: DETERMINING AN EFFECTIVE APPROACH TO REAR BIRD CHERRY-OAT APHIDS, *Rhopalosiphum padi*

4.1 Abstract

Mediums that require small spaces when rearing aphids make it possible to study these insects at a closer range. These media not only can be used to rear aphids, but also as vehicles to administer antibiotics, which might induce the death of endosymbionts, thus making it possible to study the behaviour of these insects without their nutrient providing symbiotic partners. This study tried to establish an effective manner in which aphids can be reared in smaller spaces. In addition, this study also examined the effect of two antibiotics, Rifampicin and Broad-spectrum antibiotic on bird cherry-oat aphids. The results showed that bird cherry-oat aphids found it difficult to feed on the artificial diet, which was provided in the form of parafilm sachets in a Petri dish. The aphids were unable to adapt and procreate under the parafilm sachet conditions. An average of 1.7 out of 10 aphids, which had been transferred originally, managed to survive after 72 h of incubation. The conical flasks method on the other hand, was shown to be the more superior method to rear aphids. The aphids were able to adapt and reproduce. The aphid population increased more than five fold in the same period of incubation. The conical flasks method was also used to administer the antibiotics to the aphids. Delayed growth and failure to reproduce were observed in the Rifampicin-treated aphids than in the broad-spectrum antibiotic treated aphids.

Keywords: *Rearing aphids; effective approach; growth medium; affordable; petri dish; flask method*

4.2 Introduction

Rhopalosiphum padi (bird cherry-oat aphid) is amongst the most destructive insect pests of cereal crops (Medina-Ortega *et al*., 2009; Halarewics and Gabrys, 2012). During their process of taking up phloem sap, the aphids deprive the plant of its nutrients while secreting honeydew that supports the growth of sooty moulds thus affecting the plant's photosynthetic processes (D'Arcy and Domier, 2005; Fereres and Raccan, 2015). In addition, they possibly transmit the Barley yellow dwarf virus (BYDV) virus through the phloem sieve tubes as they feed on the plant (Valenzuela and Hoffmann, 2015; Beoni *et al*., 2016; Foreman *et al*., 2016).

The phloem sap the aphid feeds on is rich in sugars and lacking in essential nutrients required by the aphid for growth and reproduction (Liadouze *et al*., 1994; Gil *et al*., 2004; Koga *et al*., 2007; Alkhedir *et al*., 2013; Machado-Assefh *et al*., 2015). This aphid has been shown to harbour primary

endosymbiotic bacteria, *Buchnera aphidicola*, which is known to assist the aphids with the synthesis of the nutrients lacking in their diet. These include essential amino acids, vitamins and lipids (Cheng *et al*., 2010, Simon *et al*., 2011, Machado-Assefh *et al*., 2015). *Buchnera aphidicola* have also been hypothesised to play a significant role in BYDV transmission by producing a chaperon protein that has a binding affinity to the virus, thus protecting it from degradation while inside the aphid (Cheng *et al*., 2010; Goncalves *et al*., 2005; Nagy *et al*., 2006; Medina-Ortega *et al*., 2009). In turn, the aphid not only plays a role of being a protective habitat to the endosymbiont, but also provides them with nutrients such as carbohydrates and non-essential amino acids (Wilkinson and Douglas 2001; Shigenobu *et al*., 2000; Alkhedir *et al*., 2003; Degnan *et al*., 2010; Hanses and Moran, 2011).

The aphid-*Buchnera* relationship is both obligate and mutualistic (Simon *et al*., 2011; Zhang *et al*., 2015) and assumed to date back more than 150 million years ago (Charles *et al*., 2011), when the then free-living *Buchnera* ancestor infected the aphid ancestor and they have been living symbiotically ever since. The relationship is known to be inseparable, as neither can survive without the other. It has, however, been shown that though they cannot be grown separately from their host (Miao *et al*., 2003; Cassone *et al*., 2015). The growth of endosymbionts can be inhibited experimentally without causing any effects on their aphid host using antibiotics (Douglas, 1992; Wilkinson and Ishikawa, 1999; Koga *et al*., 2007; Prado and Almedia, 2009; Machado-Assefh *et al*., 2015). Antibiotics facilitate bacterial cell death (bactericidal) or inhibition of bacterial growth and reproduction (bacteriostatic) (Davies, 1990; Sengupta *et al*., 2013). The resulting aposymbiotic (endosymbiotic-free) aphid can then be used as a tool to study their behaviour (Wilkinson and Ishakawa, 1999; Koga *et al*., 2007).

A number of studies have shown that antibiotics can produce aposymbiotic aphids (Koga *et al*., 2007; Cheng *et al.,* 2010; Machado-Assefh *et al*., 2015; Zhang *et al*., 2015). However, only two antibiotics have previously been successful against *B*. *aphidicola*, namely, Rifampicin (Koga *et al*., 2007; Cheng *et al*., 2010; Machado-Assefh *et al*., 2015; Zhang *et al*., 2015) and Tetracycline-Hydrochloride (HCl) (Griffiths and Beck, 1974). Rifampicin is a bactericidal that acts on both intracellular and extracellular bacteria by inhibiting DNA-dependent RNA polymerase activity resulting in the suppression of RNA synthesis (Hardman *et al*., 2001; Villain-Guillot *et al*., 2007). Tetracycline is a bacteriostatic inhibiting bacterial replication by binding to the 30S ribosomal subunit resulting in inhibition of protein synthesis (Chopra and Roberts, 2001; Chatzispyrou *et al*., 2015). Screening the effectiveness of antibiotics on aphids might aid in finding potential Biological

Control Agents (BCA) that could be used to naturally control these insects in the field. It is therefore important that the endosymbionts they carry be susceptible to a broader scale of antibiotics in order to find a wider variety of BCA.

Antibiotics have mostly been administered to aphids through artificial diets, using growth methods such as Petri dishes to rear aphids (Mittler and Dadd, 1962; Wille and Hartman, 2008; van Emden, 2009). The use of such methods have been shown to save both time and space, as it is much easier to transfer aphids to Petri dishes than to plants and take up less space compared to plants grown in pots (Wille and Hartman, 2008). However, rearing any organism in an uncontrolled environment can result in unexpected outcomes (Pianka, 2000). The organism has to first adapt to its new environment and either accept or reject it (Pianka, 2000), before it can settle and continue with its normal activities (Tares *et al*., 2013). This already means that such an organism is not performing at its optimum and observations made may therefore not reflect the true capabilities of the organism in its natural environment or conditions that are close to its natural environment. In addition, the components that make-up the artificial diet are expensive and are not universal across all aphid species. To produce aposymbiotic aphids Miao *et al*., (2003) used an approach that is much closer to the aphid's natural conditions by administering antibiotics to aphids in seedlings contained in flasks. However, this approach has not been widely adopted. In a search for finding an effective approach to control aphids, this study was conducted with the aim to establish an effective and cheaper method for rearing aphids. In addition, this study also compared the effect of two antibiotics, Rifampicin and broad-spectrum antibiotic (BSA) on bird cherry-oat aphids using the method found to be most efficient in rearing these aphids.

4.3 Materials and methods

4.3.1 Aphid maintenance

The bird cherry-oat aphids harbouring only *B*. *aphidicola* endosymbiont were collected from the cages of greenhouse 7a where they are being reared and maintained as mentioned in Chapter 3. An infested wheat pot was randomly selected from the cages and ten aphids of a similar size were collected using a dissecting microscope (SMZ800N Zoom Stereomicroscope, Nikon Optiphot Japan). The ten aphids were then transferred to two week old Hugenoot wheat cultivar seedlings and kept under the same conditions mentioned in Chapter 3 section 3.3.1. The adult aphids were removed after 24 h and collected into a 2 ml Eppendorf tube containing 500 µl of absolute ethanol

for DNA isolation. Ten nymphs were selected and transferred to new two-week old seedlings and left to produce progeny, which were used to conduct experimental procedures for this study.

4.3.2 Comparison of two approaches for rearing aphids

Two methods were screened for their effectiveness in rearing aphids in small spaces (Figure A1). The first approach was a modified Petri dish adopted from Zhang *et al*. (2015). Two-week old Hugenoot seedlings were ground into a paste using a mortar and pestle. The liquid from the Hugenoot paste was then dispensed into a stretched parafilm, which was then covered and sealed with another stretched layer of parafilm. The sachets were then placed in petri dishes layered with a moistened filter paper. Small holes were punched in the Petri dishes in order to avoid accumulation of moisture. Ten synchronised aphids were then transferred onto the parafilm sachets in the petri dishes after which the petri dishes were closed and incubated at 22^{o}C for 72 h. The second method was adopted from Miao *et al*. (2003). This included careful removal of two-week old Hugenoot seedlings, thoroughly washing off the soil from the roots and then placing these on conical flasks containing 30 ml sterile distilled water. Ten synchronised aphids were then transferred to the plants using a Camel's hair brush and incubated at 22^{o}C for 72 h. Aphids from each diet were assessed daily and their growth was measured at the end of the 72 h incubation period by counting the number of aphids recovered. In the control experiment, the aphids were reared on seedlings planted on Culterra (Johannesburg, South Africa): potting mix in 8 cm depth × 6 cm diameter pots and incubated as above.

4.3.3 Plant maintenance

Ten seeds per cultivar of the three most important cereal crops (Hugenoot-wheat, Puma-barley and Maluti-Oats) were planted into 8 cm depth × 6 cm diameter plastic pots using Cultera: professional potting mix. The crops were used to inoculate the aphids with antibiotics and evaluate the aphid's performance, by counting the number of surviving aphids at the end of each treatment. The pots were incubated as in Chapter 3 for two weeks before being infested with aphids.

4.3.4 Antibiotic treatments

The Broad-spectrum antibiotic (5 000 Units Penicillin, 5 mg Streptomycin and 5 mg Neomycin per ml) (Thermo Fisher Scientific, Johannesburg, South Africa) effect on Bird cherry-oat aphids was tested

against the widely used Rifampicin (200 µg/ml w/v) antibiotic using the flask method. Thirty millilitres of the above-mentioned antibiotics was added to single 50 ml conical flask. The control experiment contained sterile distilled water. Seedlings from the three cereal crops mentioned previously were carefully taken out from their pots and the soil was washed off from the roots. The washed seedlings were then carefully placed in the conical flasks containing the respective treatment, three seedlings per flask (Figure A2). The seedling plants were allowed a 24 h period to take-up the antibiotics and distilled water for the control plants. Ten aphids were then transferred to the plants in each prepared flask and left to feed for 72 h. To examine the long-term effect of antibiotics on the aphids, the treated aphids were transferred to two-week old healthy Hugenoot seedlings. This was done by harvesting and counting the treated aphids from all the cereal crops. Ten of the recovered aphids were counted and transferred to the healthy seedlings, separating nymphs from the adult aphids. The aphids were left to feed on the healthy Hugenoot seedlings for seven days. The remaining aphids were collected into 2 ml Eppendorf tubes containing 500 µl of absolute ethanol for DNA isolation.

4.3.5 Screening antibiotic treated aphids for primary endosymbionts

Molecular biology techniques were used to detect the presence of *B*. *aphidicola* in bird cherry-oat aphids before and after the antibiotic treatments. Total genomic DNA was extracted using the CTAB method according to Perez-Lopez and Pantoja (2014) with some modification as mentioned in Chapter 3 section 3.3.2.1. Total DNA quantity and quality was determined by monitoring $A_{260/280}$ and $A_{260/230}$ (Table A6, 7, 8) absorbance ratios using the NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, USA). Total DNA was diluted with 1× TE buffer to approximately 50 ng/µl and used as a template for the PCR amplification. The presence of primary endosymbiotic bacteria from the samples was evaluated using the *Buchnera* sp. specific primer set ApisP1_nt298-5'TTCCAG TGTGGCTGGTTA3' and 16SA1_nt1-5'AGAGTTTGA TCMTGGCTCAG3'. The reaction was carried out in a total final volume of 15 µl as described in Chapter 3 section 3.3.2.2. The amplicons were visualised and captured as described previously in Chapter 3 section 3.3.2.2.

4.3.6 Statistical Analysis

The statistical analysis for this study was calculated using Microsoft Excel 2010 student's t. test by comparing the average of each treatment at a significance value of $p \leq 0.05$.

4.4 Results

4.4.1 Comparison of the two approaches to rear aphids

The effectiveness of the two methods for rearing aphids was evaluated by measuring the growth and survival of the aphids feeding on seedling in conical flasks and parafilm sachets. A significant difference was observed in both the survival and reproduction of aphids when the two methods were compared. The aphids survived and replicated exceptionally well in the flask method (54.0 ± 6.2) compared with the parafilm sachets (1.67±1.53) (Figure 4.1). Aphids were able to reproduce on the seedlings growing in the flasks almost as much as the untreated control. They reproduced nymphs of more than five times of the aphids that were originally transferred on the plants at the beginning of the experiment. The results from the parafilm sachet method showed the complete opposite, most of them died and some of the dead ones had turned into mummies. There is a significant difference ($p \leq 0.05$) between the two methods which means that the flask method was found to be more effective over the parafilm sachet method for rearing the bird cherry-oat aphids.

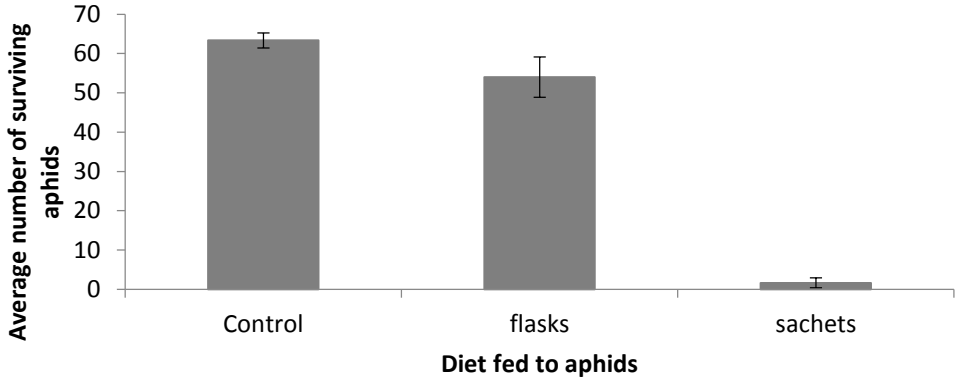

Figure 4.1: Number of surviving aphids after rearing via two different methods presented in standard error bars where n=3

4.4.2 Antibiotic administration to bird cherry-oat aphids using three cereal crops

Aphids feeding on Puma seedlings replicated most effectively on both antibiotic treatments (20.0 and 37.7 using Rifampicin and BSA, respectively) compared with the other two crops with Maluti having the least number of surviving aphids (10.3 and 26 using Rifampicin and BSA, respectively) (Figure 4.2). BSA treated aphids showed to have produced more progeny than Rifampicin treated aphids. This was shown by the average number of surviving aphids being more than that of the aphids originally transferred to the seedlings.

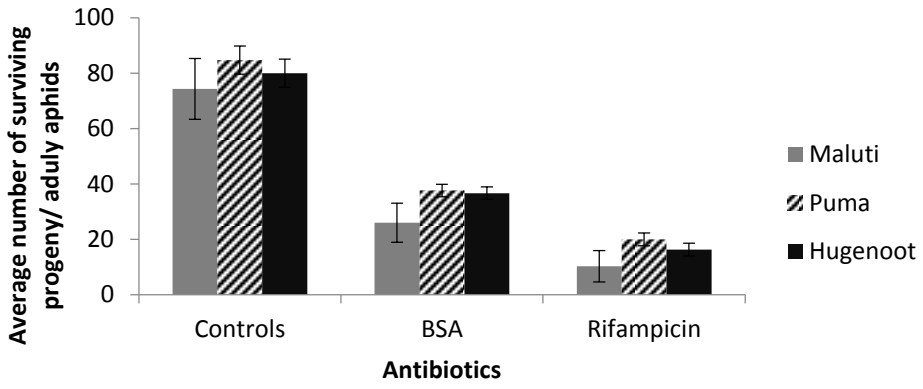

Figure 4.2: Average number of surviving aphids and their offspring recovered on the antibiotic treated plants at the end of the treatment in the flask presented in standard error bars where n=3

The reproductive rate of BSA-treated aphids was found to be higher than that of the Rifampicin-treated aphids. However, it was significantly lower than the reproductive rate of the untreated aphids. It was observed that the controls had more than seven-fold the number of aphids than originally transferred to the plants, whereas the BSA-treated aphids had more than two-fold. In the Rifampicin-treated aphids, Puma showed to have supported more aphid growth compared to the other two crops. The overall results for the three crops showed a significance difference ($p \leq 0.05$) between the two antibiotic treatments, with Rifampicin shown to be more effective by producing the least number of nymphs compared to BSA.

4.4.3 Assessment of antibiotic-treated bird cherry-oat aphids on healthy Hugenoot seedlings

4.4.3.1 Adult aphids

The survived adult aphids on both treatments continued reproducing when they were transferred to healthy wheat seedlings. Again, the aphids treated with BSA seem to have reproduced a higher number of progeny compared with that produced by the adult aphids treated with Rifampicin (Figure 4.3). The average number of surviving Rifampicin-treated adult aphids was 3.0 and they reproduced at an average number of 13.3 nymphs on healthy wheat seedlings (Figure 4.3). The average number of surviving BSA treated adult aphids was 7.3 and they produced an average number of 35.0 nymphs on the healthy wheat seedlings. Similar to the previous study, the control had a higher number of progeny compared with any antibiotic treated aphids.

Figure 4.3: Average number of the recovered adult aphids and the progeny produced after the adults were transferred from the antibiotic treatment to feed on healthy Hugenoot seedlings for seven days presented in standard error bars where n=3

4.4.3.2 Nymphs

The nymphs that were recovered after feeding on the antibiotic treated seedlings were further transferred to feed on healthy untreated wheat seedlings. The nymphs recovered from Rifampicin treatment were not capable of reproducing offspring as no progeny were observed after feeding on healthy plants for seven days from these aphids (Figure 4). Instead, the surviving aphids were smaller in size and their number had declined significantly by almost half (5.7) from the original ten aphids that were transferred to the plants (Figure 4.5C). On the other hand, the BSA treated nymphs transferred to healthy seedlings were able to grow and reproduce (Figure 4.5F). The average surviving number of aphids from the BSA treatment was 7.7 and the average number of progeny they produced was 12.0 (Figure 4.4). After antibiotic treatments were compared with the control experiment, the control experiment had much more surviving aphids (8.33 adult aphids and 38 nymphs).

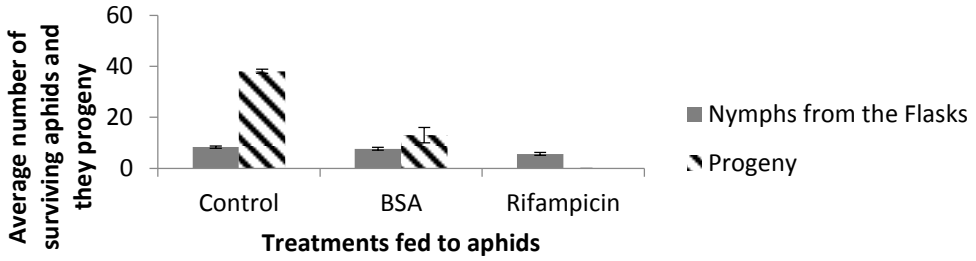

Figure 4.4: Average number of surviving aphids and their offspring after the antibiotic treated nymphs were transferred to healthy Hugenoot seedlings for seven days presented in standard error bars where n=3

4.4.4 Detection of *Buchnera aphidicola*

Molecular techniques using diagnostic PCR were employed to determine the effectiveness of Rifampicin and BSA to produce *B. aphidicola* free aphids (Figure 4.6). This was shown by the absence of the diagnostic 321 bp fragment for *B*. *aphidicola*. In the Rifampicin treated plants, *B*. *aphidicola* was only detected in the aphids that were originally transferred to feed on the antibiotic treated plants (Figure 4.6A sample 3, 5 and 7, and 4.6B sample 3, 6 and 9). However, the nymphs that these aphids produced did not contain *B*. *aphidicola* in all of the cereal crops screened (Figure 4.6A sample 4, 6 and 8) and 4.6B sample 4, 5, 7, 8, 10 and 11). Whereas in BSA treated aphids, *B*. *aphidicola* was detected not only in the aphids that were originally transferred to the flask but also in all the nymphs they produced (Figure 4.6A sample 11-16 and 4.6C sample 3-14). The presence of *B*. *aphidicola* was also confirmed in all the untreated control samples that were used, before (Figure 4.6A sample 1, 2, 9 and 10) and after (Figure 4.6B and 4.6C sample 1 and 2) antibiotic treatments.

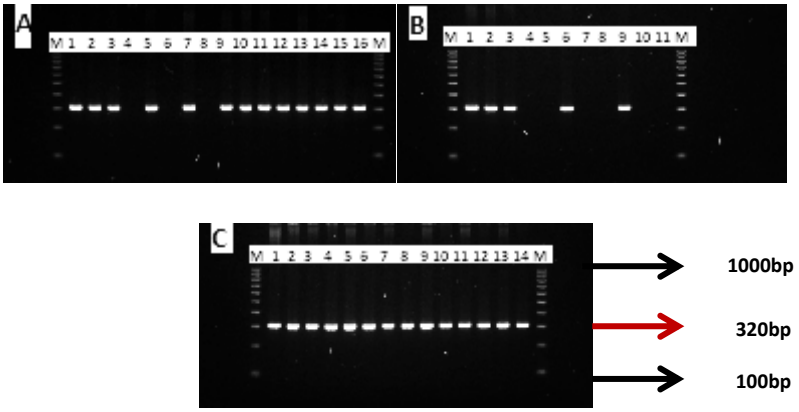

Figure 4.5: Screening for the presence of *Buchnera aphidicola* on the aphids before and after the antibiotic treatment. A-Aphids feeding on antibiotic treatment in flask, A1-8 are Rifampicin treated aphids and A9-16 are BSA treated aphids from the three cereal crops, B-Rifampicin treated aphids feeding on healthy plants and C-BSA treated plants feeding on healthy plants.

4.5 Discussion

In 2008, Willie and Hartman found aphids feeding on a detached soybean leaf to have high survival and reproduction rates compared with aphids feeding on artificial diet contained in a parafilm. Studies have shown that aphids are known to make use of different signals, starting by finding and landing on a suitable host (Lazzarotto *et al*., 2011; Suddeth and Suddeth, 2013). Once the suitable host is found, more signals come into play and these include initial plant contact and assessment of

the surface before stylet puncture (Powell *et al*., 2006). This makes aphid survival to depend on finding a suitable host in which it can feed and be able to reproduce. These insects are capable of exploring different plants, but can only exploit a plant if it is a suitable host once contact has been made (Powell *et al*., 2006). When they fail to adapt to the stresses imposed by their environments, which could be brought on by events such as not finding the suitable host or crowding, they tend to move to environments that can support both their growth and development (Tares *et al*., 2013). When conditions are favourable, the aphids maximise their energy investment on reproduction (Powell *et al*., 2006).

This study subjected the bird cherry-oat aphids to two environments. The parafilm sachets diet, which proved to be an extreme environment to what the aphids are naturally used too, and the flask diet, which was closer to the aphid's natural environment. The observations from these two diet-treatments clearly showed that the parafilm sachet diet proved to be an unsuitable environment for the aphids, as they were unable to survive and/or reproduce to the best of their ability. As soon as the aphids were transferred to the sachets on the Petri dishes, much of what seemed to be unsettled behaviour was observed. This was shown by the continuous movements they were making, from the sachet to the filter paper, across the petri dish edges to the lid. They seem to find it difficult to feed and settle onto the sachets, even after they were means were made for them to move back to their food source. They started moving around again soon after being re-placed on the sachets. The number of surviving aphids started to decrease daily to a point of having no living aphids left to be recovered on some petri dishes by the end of the experiment.

The opposite was observed for the aphids that were reared in the flasks. They settled as soon as they landed on the plants. Although aphids were not counted daily (to avoid unsettlement that might be caused by moving the mediums growing the aphids), nymphs were observed 24 h after the aphids were transferred to the flasks. An increase in their numbers was observed until the last day of the experiment. This method proved to be more efficient for rearing the bird cherry-oat aphids than the parafilm sachets, and was therefore an approach chosen to administer antibiotics to aphids.

Bacterial endosymbionts are known to respond differently to antibiotic treatments (Kohanski *et al*., 2010). Griffiths and Beck (1974) reported chlortetracycline-HCl to have produced aposymbiotic pea aphids which not only failed to reproduce but also had a delayed growth, compared with penicillin

which left aphids looking normal and capable of reproducing. Koga *et al*. (2007) also demonstrated that ampicillin and Rifampicin antibiotics have selective elimination capabilities in a dose-dependent manner over *Serratia symbiotica* and *B*. *aphidicola* survival, respectively. The dose-dependence was different between the aphid genotypes. In addition to finding an approach to rear aphids, the effectiveness of antibiotics (Rifampicin and BSA) was screened in-terms of the survival and reproduction of the bird cherry-oat aphid.

The bird cherry-oat adult aphid from both Rifampicin and BSA treatments were still capable of reproducing at the end of both experiments. The bird cherry-oat aphids proved to have more progeny under the BSA treatment compared to that under the Rifampicin treatment (Figure 4.3). However, the nymphs produced by aphids feeding on the Rifampicin treatment were noted to be relatively smaller in size compared to those produced by aphids feeding on the BSA treatment. Aphids were observed to have higher reproduction rates on Puma (barley) seedlings, while Maluti (oats) was shown to have the lowest reproduction rates. This was shown by the number of surviving aphids and the progeny produced on these seedlings (Appendix VI). This might have been due to Puma being more of a favourable host plant compared to Hugenoot and Maluti plants. Host plants have been shown to play a crucial role in the reproduction rates of insects (Taheri *et al*., 2010).

The antibiotic-treated aphids were then recovered from the treatments and transferred to feed on untreated wheat seedlings. The adult aphids recovered from both antibiotics were observed to have continued with reproducing on healthy plants, however, the Rifampicin-treated aphids produced relatively smaller progeny and in low numbers compared to the BSA-treated aphids. The progeny from the Rifampicin treatment failed to reproduce when transferred to healthy seedlings and took longer to grow compared to the progeny from the BSA-treatment which continued to reproduce on healthy seedlings. This shows that Rifampicin was more effective in delaying the growth and affecting the reproduction capabilities of bird cherry-oat aphids.

The use of molecular techniques to detect *B*. *aphidicola* after the antibiotic treatments showed the presence of the endosymbiont in the adult aphids that fed on the Rifampicin treatment. However, *B*. *aphidicola* was not detected in their progeny when they were screened with *B*. *aphidicola* specific markers (Figure 4.5A and 4.5B). The results support the findings that the growth of aphid endosymbionts can be inhibited experimentally using targeted antibiotics without causing any

effects on their aphid host (Douglas, 1992; Wilkinson and Ishikawa, 1999; Koga *et al*., 2007; Prado and Almedia, 2009; Machado-Assefh *et al*., 2015). The inhibited growth of *B*. *aphidicola* from these aphids resulted in endosymbionts failing to be transferred between the aphid generations (Cheng *et al*., 2010; Simon *et al*., 2011; Machado-Assefh *et al*., 2015). Without *B*. *aphidicola*, the progeny of the bird cherry-oat aphids were not able to replicate. The same was not observed for the BSA-treated aphids, both the adult aphids and their progeny displayed the 321 bp expected band size for *B*. *aphidicola* (Figure 4.6). *B*. *aphidicola* are vertically transferred from mother to offspring (Braendle *et al*., 2003). Unlike Rifampicin-treated aphids, which were unable to transfer *B*. *aphidicola* to their offspring, BSA-treated aphids were able to infect their offspring with the primary endosymbiont that was detected across treated aphids and their offspring which were able to grow and reproduce.

This study has shown that the use of Rifampicin on the bird cherry-oat aphids can hinder both the growth and reproduction capabilities of this aphid. The use of this antibiotic as a form of management control could result in crop production increase. However, this might be an expensive and very controversial practise, as antibiotics on a large scale would be costly and overuse can result in the development of resistance and negatively affect both the environment and human health safety. On the other hand, antibiotics are naturally synthesised by many microorganisms. For instance, Tetracycline is known to be produced by *Streptomyces* sp (Chopra *et al*., 2001). If antibiotic producing microorganisms were to be used as antagonists to the aphids instead of using the antibiotics, it might lead to some level of control especially if they were to be applied to plants at an early stage before aphid infestation occurs.

4.6 Conclusion

This study was able to rear bird cherry-oat aphids using an approach that was not only favourable in promoting aphid growth and reproduction, but also used less space and was relatively affordable. This study also showed that antibiotics have different effects on these aphids. The aphids were reared in conditions that simulate their natural habitats with the hope that it will favour both the growth and survival of the aphids. However, regardless of the conditions offered by this environment the progeny of the aphids feeding from the Rifampicin treatment failed to grow and reproduce. Whereas, the progeny produced by the aphids feeding on the BSA treatment continued to grow and reproduce. The results of this study have shown that bird cherry-oat aphids reacted differently to these antibiotics, with Rifampicin having demonstrated a negative impact on the

aphid's growth and reproduction over BSA. This antibiotic has proven on a number of occasions that it is affective against *B*. *aphidicola*. Aphids have also demonstrated that they are unable to survive and reproduce in the absence of this bacterium. It would be interesting going forward if microorganisms that naturally produce this antibiotic were to be tested on aphids as a biological control agent.

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CHAPTER 5: HOST PLANTS AS A ROUTE FOR BIRD CHERRY-OAT APHIDS, *Rhopalosiphum padi*, TO ACQUIRE SECONDARY ENDOSYMBIONTS

5.1 Abstract

Symbiotic relationships amongst bacteria and insects are common in natural environments, with bacteria using anything possible to spread themselves between different species. This is a common practise used by secondary endosymbionts. In aphids this has mostly been conducted through artificial means and therefore not giving a clear indication of how this transmission occurs or if it occurs in a field environment. This study demonstrated the ability of secondary endosymbiotic free bird cherry-oat aphid to acquire secondary endosymbionts from an infected rose grain aphid through a shared food source in two different approaches. In the first approach, *H*. *defensa* free wheat seedlings were infested with *H. defensa* infected rose grain, these were later removed and the plants were then infested with *H*. *defensa*-free bird cherry-oat aphids. The rose grain aphid was not only able to infect the plant but the newly *H*. *defensa* infected plant was also able to infect bird cherry-oat aphids with the newly obtained *H*. *defensa*. In the second approach, the *H*. *defensa* free-plants were infested with both *H*. *defensa*-free bird cherry-oat aphids and *H*. *defensa* infested rose grain aphids simultaneously. Again, the rose grain aphid was able to transmit *H*. *defensa* to both the plant and bird cherry-oat aphids.

Keyword: *Bird cherry-oat aphid; rose grain aphid; secondary endosymbionts; acquire; host plant, horizontal transmission*

5.2 Introduction

Bacteria are known to occupy almost every environmental niche on Earth (Hogan, 2010). In most cases, bacteria use these niches in order to spread across and within organisms (Strong and Davidson, 2017). The infection can either be direct, that is, from an infected organism to an uninfected organism or indirect, that is, uninfected organisms encountering an infected object. In insect species, bacterial transmission occurs through two processes, vertical transmission (in which bacteria are transmitted from mother to offspring) or horizontal transmission (where bacteria are transmitted across species through the environment or mating) (Russell and Moran, 2005; Oliver *et al*., 2010; Gonella *et al*., 2015). In order for symbiosis to occur, physical contact must be made

between the bacteria/bacteria-carrier and the host under conditions that will favour the transmission (Bright and Bulgheresi, 2010).

Horizontal transmission of bacteria usually involves secondary symbiosis and can occur on different circumstances in insects (Oliver *et al*., 2010; Gehner and Vorburger, 2012; Henry *et al*., 2013; Su *et al*., 2013; Guo *et al*., 2017; Vorburger *et al*., 2017). These include, experimentally obtained transmission, for example through oral feeding of the grapevine leafhopper with infected artificial diets (Gonella *et al*., 2015) or microinjection of infected haemolymph into aphids cavity (Russell and Moran, 2005; Lusasik *et al*., 2013). Transmission between prey and predator, for example where a parasitoid wasp transmits the endosymbiont to an uninfected aphid via stabbing after having fed from an infected aphid (Gehner and Vorburger 2012). Transmission has also been demonstrated through parental transmission between species when male and female insects mate during the overwintering season (Peccoud *et al*., 2014; Vorburger *et al*., 2017).

A great number of studies have demonstrated how secondary endosymbionts can be horizontally transmitted across aphids species (Russell and Moran, 2005; Simon *et al*., 2011; McLean *et al*., 2011; Henry *et al*., 2013; Lusasik *et al*., 2013). However this has been done through artificial means, that is, microinjection of symbionts and feeding aphids infected artificial diets. It has rarely been shown how horizontal transmission for aphids occurs and if it's possible for such to occur naturally. Especially in countries like South Africa where male aphids are rare, parasitic insects are limited and primary hosts for the overwintering processes of aphids such as those of the bird cherry-oat aphids are unattainable. Bird cherry-oat aphids are known to acquire and transmit plant viruses such as barley yellow dwarf viruses (BYDV), through feeding from the plant's phloem sap. It is likely that these insects also take-up secondary endosymbionts from their host plant through their stylets. This study was conducted with the aim to assess the ability of a single *Buchnera aphidicola* infected bird cherry-oat aphid to take-up/acquire secondary endosymbionts from a previously identified infected rose grain aphids, *Metopolophium dirhodum*, through a shared food source under conditions comparable to the field.

5.3 Materials and Methods

5.3.1 Maintenance and collection of the four South African cereal aphids

The presence of secondary endosymbionts was detected in four cereal crop aphids, namely, *Sitobion Avenae* (the English grain aphid), *Metopolophium dirhodum* (the rose grain aphid), *Diuraphis noxia* (the Russian wheat aphid-RWA) and the bird cherry-oat aphid. The aphids were collected in duplicates from greenhouse 7a at ARC-SG in Bethlehem, South Africa. The aphids are reared and maintained as previously mentioned in Chapter 3 section 3.3.1. An infested wheat pot was randomly selected from the cages and 25 healthy, mature and wingless aphids were collected for DNA isolation in 2 ml Eppendorf tubes containing 500 µl absolute ethanol, using a dissecting microscope (SMZ800N Zoom Stereomicroscope, Nikon Optiphot Japan).

5.3.2 Detecting endosymbiotic bacteria of four South African Aphid

PCR techniques were used to detect the endosymbiotic bacteria of four South African cereal crop aphids. Total genomic DNA was extracted using the CTAB method according to Perez-Lopez and Pantoja (2014) with some modification as mentioned in Chapter 3 section 3.3.2.1. Total DNA quantity and quality was determined by monitoring $A_{260/280}$ and $A_{260/230}$ (Table A9, A10, A11) absorbance ratios using the NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, USA). Total DNA was diluted to approximately 50 ng/µl using 1X TE buffer and used as a template for the PCR amplification. The presence of endosymbionts was detected using the primary and secondary eendosymbionts previously mentioned in Chapter 3. The reaction was carried out in a total final volume of 15 µl as described in Chapter 3 section 3.3.2.2. The amplicons were visualised and captured as described previously in Chapter 3 section 3.3.2.2.

5.3.3 Aphid and Plant maintenance

The Hugenoot wheat cultivar seedlings were used as a vehicle to infect the bird cherry-oat aphids with secondary endosymbionts. One seed per pot was planted in 8 cm depth × 6 cm diameter plastic pots using Culterra: professional potting mix compost. The pots were incubated as mentioned in Chapter 3 section 3.3.1 for two weeks before being infested with aphids. The rose grain and bird cherry-oat aphids used for this study were maintained on wheat cultivars. Ten aphids were selected from infested plants then transferred to two-week-old Hugenoot wheat cultivar seedlings and kept under the same conditions mentioned above. The adult aphids were removed after 24 h and

collected into 2 ml Eppendorf tube containing 500 µl absolute ethanol for DNA isolation. Ten nymphs were then selected and transferred to new two-week old seedlings and left to reproduce. The progeny was then used to infest the Hugenoot seedlings in order to assess the ability of rose grain to transmit the secondary endosymbionts to the plants and the bird cherry-oat aphids to acquire these endosymbionts from the plants.

5.3.4 Transferring and acquiring of secondary endosymbionts from rose grain aphid to plants and bird cherry-oat aphids

Bird cherry-oat aphids were infected with secondary endosymbionts using the rose grain aphid in two different techniques adopted from Capsi-Fluger *et al*. (2012). The first technique was to let the *H*. *defensa* infested rose grain aphids feed on the Hugenoot seedlings for five days after which they were harvested and collected into 2 ml Eppendorf tubes containing absolute ethanol. The rose grain aphids were then replaced with the bird cherry-oat aphids, which were also allowed five days feeding period on the assumed to be infected plants. The aphids were then harvested into 2 ml Eppendorf tubes containing absolute ethanol. The second technique to obtain secondary endosymbionts was to feed on the Hugenoot seedlings with both the rose grain aphids and bird cherry-oat aphids. The aphids were allowed five days feeding period after which they were separately harvested into 2 ml tubes containing absolute ethanol. The plants from both procedures were also harvested into 2 ml tubes before aphid infestation and after harvesting of aphids.

5.3.5 Screening for the presence of facultative endosymbionts in both aphids and plants

The ability of both the plants and bird cherry-oat aphids to acquire the secondary endosymbionts from the rose grain aphids was screened using PCR techniques as mentioned previously using *H*. *defensa* specific primer pair. For the controls, both the aphids and leaf material were collected before any interactions could occur.

5.4 Results

The presence of the primary endosymbionts, *Buchnera aphidicola*, was observed on all the four aphid species. This is shown by the presence of the expected 321 bp band size for the *Buchnera aphidicola* specific primers used (Figure 5.1A). The presence of secondary endosymbionts was only detected in the rose grain aphid, which tested positive for the *H*. *defensa* endosymbiont, (Figure

5.1B sample 5 and 6). The other three aphids, bird cherry-oat aphids (Figure 5.1B-G sample 3 and 4), RWA (Figure 5.1B-G sample 7 and 8) and the English grain aphid (Figure 5.1B-G sample 9 and 10) did not amplify the diagnostic fragments for any of the six targeted secondary endosymbionts.

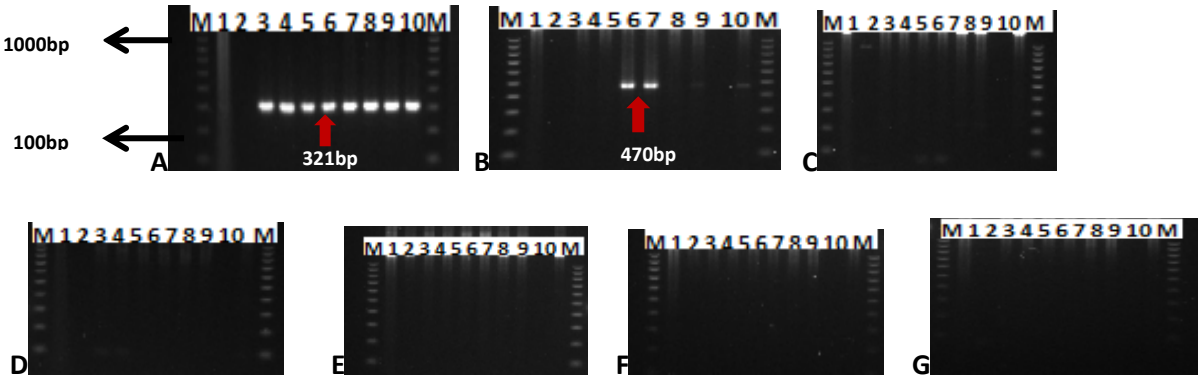

Figure 5.1: PCR detection of endosymbiotic bacteria of four South African cereal crops aphids. A - primary endosymbionts, B - G - facultative endosymbionts. B - *H. defensa*, C - *R. insecticola*, D - *Rickettsia*, E - *S. symbiotica*, F - *Spiroplasma* and G - X-type. Sample 1 and 2 are no DNA template controls, 3 and 4 - bird cherry oat aphid, 5 and 6 - rose grain aphid, 7 and 8 - RWA, 9 and 10-English grain aphid.

The plant (Figure 5.2A and 5.2B sample 1) and bird cherry-oat aphids (Figure 5.2A sample 2 and Figure 5.2B sample 4) did not amplify the diagnostic (470 bp) fragment for the presence of *H. defensa* before any interactions with the *H. defensa*-infested rose grain aphids were used for infestation (Figure 5.2A sample 3 and Figure 5.2B sample 2). The first experiment screened the ability of bird cherry-oat aphids to acquire this bacterium by horizontal transmission from *H. defensa*-infested rose grain aphid (Figure 5.2A samples 5 and 6) through a shared food source, 14 day old healthy wheat seedlings (Figure 5.2A sample 1).

At the end of the experiment, both the plant (Figure 5.2A sample 7 and 8) and bird cherry-oat aphids (Figure 5.2A sample 3 and 4) tested positive for *H. defensa*. In the second experiment, bird cherry-oat aphids fed on plants that were previously infested with *H. defensa* infested rose grain aphids (Figure 5.2B sample 2 and 6). The *H. defensa*-infested rose grain aphid not only transmitted the secondary endosymbiont to the plant (Figure 5.2B sample 3 and 7), but the bird cherry-oat

aphids (Figure 5.2B sample 5) were able to acquire the secondary endosymbiont from the infected plant as well.

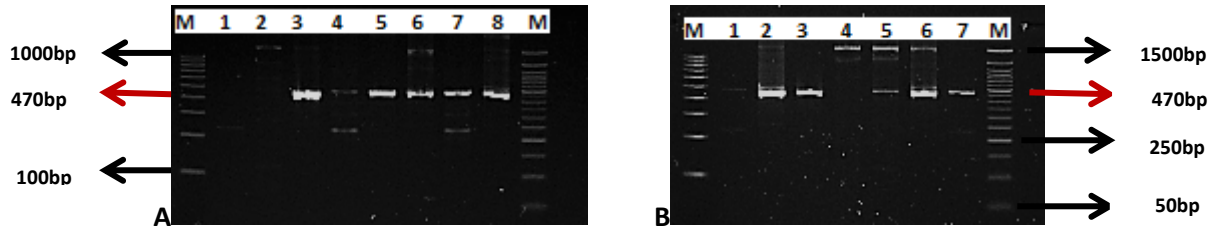

Figure 5.2: PCR detection of *H. defensa* in aphids and plants. A-both aphids feeding on the plant at the same time (samples 1, 7 and 8: Plant DNA samples; samples 2-4: bird cherry-oat aphid DNA samples; samples 5 and 6: rose grain aphid), B-bird cherry-oat aphids feeding on previously rose grain infested plants (samples 1, 3 and 7: plant DNA samples; samples 4 and 5: bird cherry-oat aphid DNA sample; samples 2 and 6: H. *defensa* infested rose grain aphid DNA samples)

5.5 Discussion

The environment provides vast and inconstant conditions for persistent and intimate interactions to occur (Bennett, 2013). One of these is the three-way interaction between plants, microbes and insects in which the plant plays a go-between role for the microbes and insects (Biere and Tack, 2013). These interactions may result in insects acquiring endosymbionts which may shape the way they interact with their environment, be it the ability to invade new host plants and/or avoid/resist their enemies and adapting to a constant changing climate (Wiescher *et al*., 2011; Biere and Bennedett, 2013). These benefits are mostly known to be provided to the aphid host by their secondary endosymbionts. Unlike their primary endosymbiont counterparts that have an obligate relationship with their host, the incidence of these particular endosymbionts is known to occur in a sporadic manner across their hosts (Oliver *et al*., 2010; Guo *et al*., 2017; Li *et al*., 2017; Vorburger *et al*., 2017).

In this study, the presence of *B. aphidicola* known as primary endosymbiotic bacteria was detected in all of the four species of aphids screened. Only the rose grain aphid, of the four tested aphid species, tested positive for a secondary endosymbiont. The rose grain aphid (Figure 5.1B sample 5 and 6) carried an extra endosymbiont, namely *H. defensa*, in addition to its primary endosymbiont. These results further demonstrate the variability in the occurrence of secondary endosymbionts

amongst aphid species. A number of studies have shown that in the absence of aphid's natural enemies and variation of environmental conditions, the presence of secondary endosymbionts tends to be costly to their aphid host (Oliver *et al*., 2006; Simon et al., 2011; Vorburger and Gouskov, 2011; Dykstra *et al*., 2014; Polin *et al*., 2014). The glasshouse conditions under which these aphids were maintained offered environmentally friendly conditions to the aphids and they might not need the protection offered by these endosymbionts. The presence of *H*. *defensa* in the rose grain aphid may be to assist the aphids in other ways that does not involve protection against natural enemies or fluctuating temperatures. This needs further investigations to understand why the aphid still harbours this endosymbiont under favourable conditions.

Secondary endosymbionts have been shown to occur in different tissues within the aphid, which can be either extracellular or intercellular (Oliver *et al*., 2010; Li *et al*., 2017). Occurrences of horizontal transmission of secondary endosymbionts has been shown to result from a number of interactions between insects (Russell and Moran, 2005; Simon *et al*., 2011; McLean *et al*., 2011; Henry *et al*., 2013; Lusasik *et al*., 2013; Guo *et al*., 2017; Li *et al*., 2017). Le Clec'h *et al*. (2013) showed *Wolbachia* to be passed amongst different species through predation and cannibalism, *Wolbachia* was able to be ingested by *Porcellio dilatatus* and *Armadillidium vulgare* after they had preyed on a *Wolbachia* infected *A*. *vulgare*. *Wolbachia* was shown to be able to cross and survive the intestinal barriers of these predators and be passed on to other hosts. Capsi-Fluger *et al*. (2011) also demonstrated plant mediated horizontal transmission of *Rickettsia* from infected host to the plant's phloem and then into a previously uninfected whitefly.

This study validated the ability of bird cherry-oat aphids (Figure 5.1) to acquire *H*. *defensa* from the infected rose grain aphids (Figure 5.1B, sample 5 and 6) through a shared uninfected host plant (Figures 5.2A and 2B sample 1) using two different experimental approaches. At the end of both experiments, previously *H*. *defensa* free plants and bird cherry-oat aphids were found to have acquired the secondary endosymbiont from the infected rose grain aphid (Figure 5.2). In addition, previously *H*. *defensa*-free bird cherry-oat aphids were capable of acquiring *H*. *defensa* from the previously infested plant without the presence of infested aphids (Figure 2B). This shows how secondary endosymbionts can be transferred between plants and aphids. This exchange of secondary endosymbionts was observed in a controlled environment. Natural environments provide a vast diversity in bacteria, including ones that have not been targeted in this study, which could also be partaking in this interactive exchange between aphids, plants and other insects.

5.6 Conclusion

This study has shown that aphids are capable of acquiring secondary endosymbionts through a shared food source. By being able to pass across aphids tissues and escape the plants defence systems, secondary endosymbionts have shown that they are capable of using different methods as their vehicles to spread across different species. They have also shown that they do not need their host to be physically present for the infection to occur. The infection can occur both direct resulting from the presence of both infected and uninfected host or indirectly in the absence of the infected host which had previously invaded a medium that an uninfected host can easily come across. The role of secondary endosymbionts has been mostly studied on the pea aphids. It would be interesting for future studies to investigate whether the same secondary endosymbionts provide the same functions in other aphid hosts. For instance, the role *H*. *defensa* play in the rose grain aphid in the absence of unfavourable circumstances.

5.7 References

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CHAPTER 6: CONCLUDING REMARKS

The questions posed before this study commenced have been answered. The South African bird cherry-oat aphid was found to harbour the obligate endosymbiotic bacteria, *Buchnera aphidicola*. Although not much variation was observed for the secondary endosymbionts, two of the seven samples screened, tested positive for *H*. *defensa*. This study also demonstrated that antibiotics have different effects on the bird cherry-oat aphid. Rifampicin was shown to impact the growth and reproduction of the aphid negatively, whereas BSA had the opposite effect as the aphid continued growing and reproducing similar to the untreated control. This study has also shown that the bird cherry-oat aphid is capable of acquiring secondary endosymbionts from its surrounding environment.

It must however also be remembered that the field, where these insects thrive, is very diverse. These insects are exposed to numerous microorganisms that they can acquire. However, this study has shown that *B*. *aphidicola* is the key that keeps the aphid fully functioning. In the absence of this endosymbiont, the aphid can barely survive. This gives an indication that these pests can be manipulated through eliminating the symbiotic association they have with this particular bacterium. Numerous studies have shown that these endosymbionts are sensitive to two antibiotics, namely, Rifampicin and Tetracycline. This study has also shown that Rifampicin antibiotic have a greater impact on the South African bird cherry-oat aphid over the Broad-spectrum antibiotic. The aphids took longer periods to develop and two subsequent generations later were sterile. However, this was done under controlled conditions, in which there were no natural enemies introduced and aphids screened only harboured the primary endosymbionts. Although this study was conducted under controlled conditions, this study has shown that targeting aphid microbiota can suppress bird cherry-oat aphids. However, as effective as it has demonstrated, this approach has its disadvantages such as overuse of antibiotics results in the development of resistance, costs of antibiotics and the feasibility of applying them in a field. Additionally, the negative adverse effects the overuse of antibiotics might have on the environmental and human. Since antibiotics are known to be produced mainly by microorganisms in nature. The use of such organisms in the field, especially during the early stages of the plant development, as a potential BCA could result in an effective management strategy for this aphid. Once treated, the aphid should not be able to grow and reproduce in amounts that would result in a significant economic impact for the farmer. Investigations to assess the ability of Rifampicin producing microorganisms to actively control the bird cherry-oat aphids are necessary.

APPENDIX

Reagent Preparation

1. **0.5 M EDTA**

Ingredients for 10 ml:

1.861 g disodium EDTA•$2H_2O$ EDTA

0.2 g Sodium Hydroxide (NaOH)

Dissolve in 8 ml water, add 00.2 g NaOH pellets and stir vigorously until everything dissolves. Bring the volume to 10 ml by adding the 2 ml remaining water and sterilise by filtering in 0.2 µM filter membrane

2. **5 M NaCl**

Ingredients for 35 ml:

10.23 g NaCl

Dissolve in 35 ml distilled water and sterilise by filtering in 0.2 µM filter membrane

3. **1 M Tris pH 8 solution**

Ingredients for 35 ml:

1.855 g of Tris-base (Tris (hydroxymethyl) aminomethane)

3.108 g of Tris-HCl

Dissolve in 35 ml distilled water and sterilise by filtering in 0.2 µM filter membrane

4. **CTAB extraction buffer**

Ingredients for 100 ml:

2 % CTAB (2.0 g)

100 mM Tris pH 8 (10 ml of 1.0 M sol)

20 mM EDTA (4 ml of 0.5 M sol)

1.4 M NaCl (28 ml of 5 M NaCl)

57.8 ml of distilled water

0.2 % Beta mercaptoethanol Add just before use (200 µl)

Dissolve all reagents in 57.8 ml water

5. **10 X TBE (Tris-borate-EDTA) buffer:**

Ingredients for 1 L:

108 g Tris-Base

55 g Boric acid

7.5 g disodium salt EDTA

Dissolve the Tris, boric acid and EDTA in 1 L distilled water

6. **TE (Tris-EDTA) buffer:**

Ingredients for 100 ml:

1 ml 1 M Tris

0.2 ml 0.5 M EDTA

Add the solutions to 98.8 ml distilled water. Sterilise by filtering through a 0.2 µM filter membrane

7. **Chloroform: Isoamyl alcohol (24:1) 25 ml**

Mix 24 ml of chloroform with 1 ml isoamyl alcohol in a 50 ml Schott bottle.

8. **70% Ethanol 100 ml**

Add 30 ml distilled water to 70 ml absolute ethanol.

Chapter 3

Table A1: DNA concentrations for the seven bird cherry-oat aphids used to detect endosymbionts

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	*E. coli*	ARC	1/25/2017 13:33	2124.3	ng/µl	42.487	22.586	1.88	1.85	DNA	50
2	BCOAC1R1	ARC	1/25/2017 13:34	1727.4	ng/µl	34.548	17.912	1.93	1.94	DNA	50
3	BCOAC1R2	ARC	1/25/2017 13:34	1903.7	ng/µl	38.074	19.86	1.92	1.94	DNA	50
4	BCOAVR1	ARC	1/25/2017 13:35	2037.1	ng/µl	40.742	20.995	1.94	1.99	DNA	50
5	BCOAVR2	ARC	1/25/2017 13:35	1611.3	ng/µl	32.226	16.68	1.93	1.98	DNA	50
6	BCOASC1R1	ARC	1/25/2017 13:36	1402.1	ng/µl	28.043	14.705	1.91	1.93	DNA	50
7	BCOASC2R2	ARC	1/25/2017 13:36	1345.6	ng/µl	26.912	13.918	1.93	1.95	DNA	50
8	BCOASC2R1	ARC	1/25/2017 13:36	1451.7	ng/µl	29.033	15.095	1.92	1.98	DNA	50
9	BCOASC2R2	ARC	1/25/2017 13:37	1588.7	ng/µl	31.774	15.845	2.01	2.15	DNA	50
10	BCOASC1R1	ARC	1/25/2017 13:37	1424.5	ng/µl	28.49	14.251	2	2.12	DNA	50
11	BCOASC1R2	ARC	1/25/2017 13:38	6055.7	ng/µl	121.114	60.715	1.99	2.14	DNA	50
12	BCOAC1R1	ARC	1/25/2017 13:38	3042.9	ng/µl	60.857	30.906	1.97	2.12	DNA	50
13	BCOAC1R2	ARC	1/25/2017 13:39	2890.5	ng/µl	57.809	29.04	1.99	2.04	DNA	50
14	BCOAMR1	ARC	1/25/2017 13:39	4057.3	ng/µl	81.147	40.356	2.01	2.02	DNA	50
15	BCOAMR2	ARC	1/25/2017 13:39	1093.6	ng/µl	21.873	11.511	1.9	1.91	DNA	50

Chapter 4

Table A2: Recovered aphids after feeding on the flask and sachets diet for three days

Hugenoot replicas	State of aphids	Flask	Sachets
R1	Adult aphids	9	3
	Progeny	45	0
	Winged-Alive	5	0
	Dead	7	6
	Mummy	4	1
	Winged-Dead	2	0
R2	Adult aphids	10	0
	Progeny	43	0
	Winged-Alive	3	0
	Dead	4	10
	Mummy	6	0
	Winged-Dead	0	0
R3	Adult aphids	7	2
	Progeny	37	0
	Winged-Alive	3	0
	Dead	10	5
	Mummy	4	3
	Winged-Dead	3	0
Control 1	Adult aphids	9	10
	Progeny	56	59
	Winged-Alive	1	3
	Mummy	1	2
	Dead	5	5
	Winged-Dead	1	5
Control 2	Adult aphids	8	9
	Progeny	51	54
	Winged-Alive	3	0
	Mummy	5	6
	Dead	1	4
	Winged-Dead	0	0
Control 3	Adult aphids	8	9
	Progeny	53	58
	Winged-Alive	1	1
	Mummy	6	8
	Dead	2	6
	Winged-Dead	1	0

Notes: The average number of surviving aphids was calculated as the examples below for all the experimental treatments. The number of surviving aphids was obtained by adding all the surviving number of aphids that is the adult aphids, their progeny and the winged alive aphids. The only surviving aphids obtained for this diet were few of those originally transferred to the petri dishes, therefore there was no need for adding up the surviving number of aphids per replica as only one category, that is adult aphids, had surviving aphids. Only the control had surviving aphids in all the categories where some form of survival was expected.

Sum of surviving aphids in flask diet

R1= surviving adult aphids + surviving progeny + surviving winged aphids

R1=9+45+5=59

Table A3: Recovered aphids reared on three cereal crops in the conical flask containing BSA and Rifampicin treatments

Cereal crops	State of Aphids	Rifampicin			BSA		
		R1	R2	R3	R1	R2	R3
Maluti	Transferred aphids	7	8	4	8	6	5
	Progeny	4	4	3	27	19	10
	Winged-A	1	0	1	0	2	1
	Winged-D	1	0	0	0	0	0
	Dead	1	0	0	2	1	4
	Mummy	2	6	5	5	3	5
Puma	Transferred aphids	7	5	4	6	6	9
	Progeny	7	16	15	25	29	36
	Winged-A	1	3	2	0	2	0
	Winged-D	0	0	2	0	0	1
	Dead	1	2	1	0	0	5
	Mummy	4	5	4	6	4	7
Hugenoot	Transferred aphids	5	4	4	9	6	8
	Progeny	13	11	12	33	28	21
	Winged-A	0	0	0	1	1	3
	Winged-D	0	1	1	0	0	0
	Dead	3	2	1	2	2	3
	Mummy	3	5	2	11	4	3
Control (Maluti)	Transferred aphids	8		8		9	
	Progeny	63		59		67	
	Winged-A	5		1		3	
	Winged-D	0		0		0	
	Dead	2		4		0	
	Mummy	4		7		3	
Control (Puma)	Transferred aphids	9		9		9	
	Progeny	75		69		72	
	Winged-A	3		4		1	
	Winged-D	0		0		0	
	Dead	0		2		3	
	Mummy	4		4		2	
Control (Hugenoot)	Transferred aphids	9		8		9	
	Progeny	69		65		67	
	Winged-A	5		4		4	
	Winged-D	0		0		0	
	Dead	2		4		0	
	Mummy	4		3		4	

Table A4: Recovered adult aphids transferred to healthy plants after antibiotic treatment

Hugenoot replicas	State of aphids	Rifampicin	BSA
R1	Transferred aphids	2	7
	Progeny	6	31
	Winged-A	2	3
	Dead	2	2
	Mummy	8	2
	Winged-D	0	0
R2	Transferred aphids	1	6
	Progeny	10	23
	Winged-A	3	1
	Dead	6	0
	Mummy	3	7
	Winged-D	0	0
R3	Transferred aphids	6	9
	Progeny	17	46
	Winged-A	2	2
	Dead	4	2
	Mummy	4	0
	Winged-D	0	0
Control 1	Transferred aphids	8	
	Progeny	55	
	Winged-A	2	
	Winged-D	3	
	Dead	5	
	Mummy	10	
Control 2	Transferred aphids	8	
	Progeny	57	
	Winged-A	3	
	Winged-D	0	
	Dead	5	
	Mummy	7	
Control 3	Transferred aphids	7	
	Progeny	53	
	Winged-A	3	
	Winged-D	1	
	Dead	6	
	Mummy	4	

Figure A1: Experimental set-up for comparing two approaches to rear Bird cherry-oat aphids (the petri dish method, conical flask method and the control)

Table A5: Recovered antibiotic treated nymphs that were transferred to healthy plants

Hugenoot replicas	State of aphids	Rifampicin	BSA
R1	Transferred nymphs	5	8
	Progeny	0	12
	Winged-A	0	0
	Dead	4	3
	Mummy	1	4
	Winged-D	1	0
R2	Transferred nymphs	6	7
	Progeny	0	9
	Winged-A	0	0
	Dead	1	1
	Mummy	3	4
	Winged-D	0	3
R3	Transferred aphids	6	8
	Progeny	0	14
	Winged-A	0	1
	Dead	1	3
	Mummy	3	2
	Winged-D	0	2
Control 1	Transferred nymphs	8	
	Progeny	36	
	Winged-A	1	
	Winged-D	0	
	Dead	3	
	Mummy	5	
Control 2	Transferred nymphs	8	
	Progeny	39	
	Winged-A	0	
	Winged-D	2	
	Dead	3	
	Mummy	4	
Control 3	Transferred nymphs	9	
	Progeny	35	
	Winged-A	3	
	Winged-D	0	
	Dead	5	
	Mummy	7	

Figure A2: Experimental set-up for assessing the effectiveness of BSA and Rifampicin on bird cherry-oat aphids

Table A6: DNA concentration of bird cherry-oat aphids recovered from the three antibiotic-treated cereal crops

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	RCA	ARC	10/21/2017 14:42	669.6	ng/µl	13.393	6.862	1.95	2	DNA	50
2	RCB	ARC	10/21/2017 14:43	903.3	ng/µl	18.066	9.066	1.99	1.97	DNA	50
3	HR1	ARC	10/21/2017 14:36	1055.8	ng/µl	21.117	10.141	2.08	2.05	DNA	50
4	HR2	ARC	10/21/2017 14:37	506.3	ng/µl	10.125	5.187	1.95	1.71	DNA	50
5	PR1	ARC	10/21/2017 14:38	1339.6	ng/µl	26.791	13.055	2.05	2.03	DNA	50
6	PR2	ARC	10/21/2017 14:38	228.8	ng/µl	4.576	2.394	1.91	1.63	DNA	50
7	OR2	ARC	10/21/2017 14:38	1684.9	ng/µl	33.698	16.111	2.09	2.09	DNA	50
8	OR3	ARC	10/21/2017 14:39	575.1	ng/µl	11.503	5.763	2	1.87	DNA	50
9	BCA	ARC	10/21/2017 14:42	2526	ng/µl	50.52	24.466	2.06	2.06	DNA	50
10	BCB	ARC	10/21/2017 14:42	3869.1	ng/µl	77.381	37.759	2.05	2.05	DNA	50
11	HR1	ARC	10/21/2017 14:39	2269.1	ng/µl	45.382	21.337	2.13	2.23	DNA	50
12	HR2	ARC	10/21/2017 14:40	1283.3	ng/µl	25.666	12.676	2.02	2.1	DNA	50
13	PR1	ARC	10/21/2017 14:40	2737	ng/µl	54.74	26.138	2.09	2.2	DNA	50
14	PR2	ARC	10/21/2017 14:40	1114.2	ng/µl	22.284	1.99	2.03		DNA	50
15	OR1	ARC	10/21/2017 14:41	1802	ng/µl	36.041	17.012	2.12	2.16	DNA	50
16	OR2	ARC	10/21/2017 14:41	617.6	ng/µl	12.352	6.293	1.96	1.83	DNA	50

Table A7: DNA concentration of bird cherry-oat aphids from Rifampicin antibiotic treatment

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	RHCA	ARC	10/21/2017 14:42	675.5	ng/µl	13.51	6.878	1.96	1.97	DNA	50
2	RHCB	ARC	10/21/2017 14:43	903.3	ng/µl	18.066	9.066	1.99	1.97	DNA	50
3	HA	ARC	10/21/2017 14:45	341.3	ng/µl	6.825	3.395	2.01	1.78	DNA	50
4	HA-B	ARC	10/21/2017 14:55	157.1	ng/µl	3.141	1.73	1.82	1.3	DNA	50
5	HB	ARC	10/21/2017 14:54	263.3	ng/µl	5.267	2.991	1.76	1.24	DNA	50
6	PA	ARC	10/21/2017 14:53	215.6	ng/µl	4.312	2.338	1.84	1.32	DNA	50
7	PA-B	ARC	10/21/2017 14:55	45.3	ng/µl	0.905	0.64	1.41	0.58	DNA	50
8	PB	ARC	10/21/2017 14:54	98.5	ng/µl	1.97	1.202	1.64	0.91	DNA	50
9	OA	ARC	10/21/2017 14:54	223.5	ng/µl	4.47	2.431	1.84	1.31	DNA	50
10	OA-B	ARC	10/21/2017 14:55	15.7	ng/µl	0.314	0.184	1.71	0.68	DNA	50
11	OB	ARC	10/21/2017 14:55	30	ng/µl	0.6	0.404	1.49	0.55	DNA	50

Table A8: DNA concentration of bird cherry-oat aphids from BSA-treatment

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	BHCA	ARC	10/21/2017 14:56	5218.1	ng/µl	104.363	52.779	1.98	1.73	DNA	50
2	BHCB	ARC	10/21/2017 14:42	3869.1	ng/µl	77.381	37.759	2.05	2.05	DNA	50
3	HA	ARC	10/21/2017 14:57	1439.9	ng/µl	28.798	14.523	1.98	1.84	DNA	50
4	HA-B	ARC	10/21/2017 14:58	1556.6	ng/µl	31.133	15.775	1.97	1.93	DNA	50
5	HB	ARC	10/21/2017 14:59	1638.1	ng/µl	32.761	16.313	2.01	2.07	DNA	50
6	HB-B	ARC	10/21/2017 15:00	848.2	ng/µl	16.965	8.589	1.98	2.04	DNA	50
7	PA	ARC	10/21/2017 14:57	851.9	ng/µl	17.037	8.657	1.97	1.79	DNA	50
8	PA-B	ARC	10/21/2017 14:58	538.5	ng/µl	10.769	5.504	1.96	1.78	DNA	50
9	PB	ARC	10/21/2017 14:59	1514.3	ng/µl	30.285	15.241	1.99	2.01	DNA	50
10	PB-B	ARC	10/21/2017 15:00	443.8	ng/µl	8.877	4.828	1.84	1.37	DNA	50
11	OA	ARC	10/21/2017 14:58	745.6	ng/µl	14.912	8.017	1.86	1.43	DNA	50
12	OA-B	ARC	10/21/2017 14:58	631.1	ng/µl	12.622	6.643	1.9	1.65	DNA	50
13	OB	ARC	10/21/2017 14:59	1243.9	ng/µl	24.878	13.147	1.89	1.49	DNA	50
14	OB-B	ARC	10/21/2017 15:00	401.4	ng/µl	8.028	4.184	1.92	1.72	DNA	50

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Table A9: DNA concentration of four South African cereal crops

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	BCOA	ARC	1/25/2017 13:36	1345.6	ng/µl	26.912	13.918	1.93	1.95	DNA	50
2	BCOA	ARC	1/25/2017 13:36	1451.7	ng/µl	29.033	15.095	1.92	1.98	DNA	50
3	RGA	ARC	1/25/2017 13:37	1588.7	ng/µl	31.774	15.845	2.01	2.05	DNA	50
4	RGA	ARC	1/25/2017 13:37	1424.5	ng/µl	28.49	14.251	2	2.12	DNA	50
5	RWA	ARC	1/25/2017 13:38	6055.7	ng/µl	121.114	60.715	1.99	2.14	DNA	50
6	RWA	ARC	1/25/2017 13:38	3042.9	ng/µl	60.857	30.906	1.97	2.12	DNA	50
7	EA	ARC	1/25/2017 13:39	2890.5	ng/µl	57.809	29.04	1.99	2.04	DNA	50
8	EA	ARC	1/25/2017 13:39	4057.3	ng/µl	81.147	40.356	2.01	2.02	DNA	50

Table A10: DNA concentration of bird cherry-oat and rose grain aphids feeding at the same time on Hugenoot wheat cultivar

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	PC	ARC	11/21/2017 12:12	830.4	ng/µl	16.608	8.53	1.95	2.27	DNA	50
2	BC	ARC	11/21/2017 12:13	6272.6	ng/µl	125.451	63.726	1.97	2.12	DNA	50
3	RGA1	ARC	11/21/2017 12:13	6780.5	ng/µl	135.611	70.355	1.93	2.06	DNA	50
4	PPRC	ARC	11/21/2017 12:16	833.8	ng/µl	16.676	8.479	1.97	2.28	DNA	50
5	PPR3	ARC	11/21/2017 12:16	1196.7	ng/µl	23.934	12.215	1.96	2.02	DNA	50
6	BPR3	ARC	11/21/2017 12:24	700.4	ng/µl	14.008	7.484	1.87	1.99	DNA	50
7	RPRC	ARC	11/21/2017 12:19	2090.6	ng/µl	41.813	21.707	1.93	1.82	DNA	50
8	RPR3	ARC	11/21/2017 12:21	1694.1	ng/µl	33.881	17.879	1.9	1.85	DNA	50

Table A11: DNA concentration of bird cherry-oat aphids, rose grain aphids and Hugenoot seedling before and after infection with *Hamiltonella defensa* infected Rose grain aphids

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	PHRR6	ARC	11/21/2017 12:18	5634.8	ng/µl	112.695	57.743	1.95	1.89	DNA	50
2	2ARHC	ARC	11/21/2017 12:14	2214.3	ng/µl	44.287	23.434	1.89	1.96	DNA	50
3	2PHRR6	ARC	11/21/2017 12:16	778.3	ng/µl	15.567	7.923	1.96	2.2	DNA	50
4	BC2	ARC	11/21/2017 12:16	9777.8	ng/µl	195.555	97.447	2.01	2.16	DNA	50
5	BPR2	ARC	11/21/2017 12:19	1120.5	ng/µl	22.41	11.59	1.93	2.15	DNA	50
6	PRRC	ARC	11/21/2017 12:24	750.9	ng/µl	15.018	6.517	2.03	2.07	DNA	50
7	PABR2	ARC	11/21/2017 12:17	5390.7	ng/µl	107.813	54.363	1.98	2.26	DNA	50

Sequences of the samples sent for sequencing to confirm the PCR positive results for *B. aphidicola* and *H. defensa*

Buchnera aphidicola

C1R2_16SA1

CGCACCCTACACATGCAAGTCGAGCGGCAGCGAAGAAGCTTGCTTTCTTGCGGCGAGCGGCAAACGGTGAGA
ATATCTGGGGATCTACCCAAAAGAGGGGGATAACTACTAGAAATGGTAGCTAATACCGCATAAAGTTGAAAA
ACCAAAGTGGGGGATCTTTTTAAGACCTCATGCTTTTGGATGAACCCAGACGAGATTAGCTTGTTGGTAAGGT
AAAAGCTTACCAAGGCCACGATCTCTAGCTGGTCTGAAGGATAACCAGCCACACTGAAA

C1R2_ApisP1

TAACGGCCTGGTAAGCTTTTACCTTACCAACAAGCTAATCTCGTCTGGGTTCATCCAAAAGCATGAGAAAAAA
AAAGATCCCCCACTTTGGTTTTTCAACTTTATGCGGATTAGCTACCATTTCTAGTAGTTATCCCCCTCTTTTGGGT
AGATCCCCAGATATTACTCACCCGTTTGCCGCTCGCCGACAAGAAAGCAAGCTTTCTTTCGCTGCCGCTCGACT
TGCATGTGTTAGGCTTGCCGCCAGCGTTCAATCTGAGCCATGATCAAACTCT

Hamiltonella defensa

RGA_10F

CGTAAGTCGACGGCATCGAGYGATCGCAGTTTACTGAGTTCATGTCGGCGAGCGGCSGACGGGTGAGTAAAG
TCTGGGAATCTGGCCGAAGGAGGGGGATAACTGCTGGAAACGGCAGCTAATACCGCATGAAGTCGCGAGAC
CAAAGTGGGGGACCTTCGGGCCTCACGCCTTCGGATGAGCCCAGATGAGATTAGCTGGTAGGTAAGGTAAAG
GCTTACCTAGGCGACGATCTCTAGCGGGTCTGAGAGGATAGCCCGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCACGTGTGT
GAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGCGATAAATGCGAATACCATTT

RGA_T419R

GAAGCCTTTACACCCGAAGGCCTTCTTCACACGTGGCATGGCTGCATCAGGCTTTCGCCCATTGCAAACCCCAC
TGCTGCCTCCCGTGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCGGGCTATCCTCTCAGACCCGCTAGAGA
TCGTCGCCTAGGTAAGCCTTTACCTTACCTACCAGCTAATCTCATCTGGGCTCATCCGAAGGCGTGAGGCCCGA
AGGTCCCCCACTTTGGTCTCGCGACTTCATGCGGTATTAGCTGCCGTTTCCAGCAGTTATCCCCCTCCTTCGGCC
AGATTCCCAGACTTTACTCACCCGTCCGCCGCTCGCCGACATGAACTCAGTAAACTGCGATCCTCGATGCCGCT
CGACTTGCATGTGTTAAGCTGCCACCAGCGTTCAATCTGAGCCATGATCAAACAA