

Endophytic establishment of *Beauveria bassiana* in wheat (*Triticum aestivum*) and its impact on *Diuraphis noxia*

LF Motholo



Orcid.org 0000-0002-9423-4777

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Promoter:

Prof MMO Thekiso

Co-promoter:

Prof TJ Tsilo

Co-promoter:

Dr JL Hatting

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DEDICATION

This thesis is dedicated to my family, my husband Mr Khateane Gedion Motholo for his unconditional support and generous encouragements and to our lovely daughters Tekano Cheryl Motholo and Lielelo "Lilly" Carol Motholo for affording me their family time to carry out this study.

My parents who rendered an endless love and support that has sustained me throughout my life.

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My two late sisters, Limakatso and Malehlohonolo, I know you would have been with me through all the hard times of this study.

I LOVE YOU ALL!



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I HOPE TO MAKE YOU PROUD

PREFACE

The experimental work presented in this thesis was conducted within the two institutions as: development and isolation of fungal strains which were performed in the Insect Pathology Laboratory, Plant Protection Department, Agricultural Research council of South Africa (ARC) – Small Grain, Bethlehem, under the supervision of Dr Justin Hatting. Molecular experiments were conducted in both North West University (NWU) Molecular Parasitology and Zoonosis Research Group laboratory, Unit of Environmental Sciences and Mamangement and the ARC – Small Grain Biotechnology laboratories under the supervision of Prof Oriel Thekisoie and Prof Toi Tsilo, respectively. This thesis represents an original work executed by the author and has not otherwise been submitted in any form for any degree to any tertiary institution. Where other people's work has been used, due reference is made in the text.

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(Philippians 1:6)*

ABSTRACT

The entomopathogenic fungus (EPF) *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) is globally known to infect a wide range of insect hosts, including aphids. The current study investigated possibilities for endophytic establishment of the EPF *B. bassiana* in wheat (*Triticum aestivum* L) (Poaceae) and explore its biocontrol potential against the Russian wheat aphid (RWA), *Diuraphis noxia* Kurdjumov (Hemiptera: Aphididae). Twelve indigenous *B. bassiana* isolates were pre-screened against *Galleria mellonella* larvae and the virulent strain (PPRI 7598) was selected based on >90% of insect mortality. PPRI 7598 was compared with PPRI 7861 (commercial strain) for pathogenicity against *D. noxia*. PPRI 7598 strain was further passaged through *D. noxia* and wheat cultivar Tugela (designating "IN" and "PL" isolates, respectively), from which wheat seedlings were inoculated with 1×10^8 conidia ml^{-1} using three inoculation techniques. Endophytic colonisation of five cultivars was evaluated post inoculation. Furthermore, the endophytic effect was evaluated on the aphid population growth with intrinsic rate of increase, plant response towards RWA herbivory, plant damage rate post infestation with RWA and impact on the aphid masses (g/plant). The *B. bassiana* strains used in this study were therefore confirmed with PCR assay using *B. bassiana* species-specific primers. The two *B. bassiana* strains were proved to be pathogenic to *D. noxia*, however PPRI 7598 caused significantly higher mortality of 55.64% than PPRI 7861 (43.1%). PPRI 7598 "IN" isolate also recorded the highest RWA mortality as compared to 50% by PPRI 7598 PL. Also, PPRI 7598 IN showed significantly higher (29.74) mean level of endophytic colonisation of seedlings than the PL isolate (26.13%). The highest levels of colonisation were 44.15% and 38.59%, recorded in roots with PPRI 7598 IN and PPRI 7598 PL, respectively. Overall, endophytic *B. bassiana* improved plant growth by 71% average over the controls.

The significant effect of the endophyte on RWA population reduced the aphid net reproductive rate by 14 nymphs female^{-1} , although the intrinsic rate of population increase was not significant. The endophyte presence significantly decreased the aphid mass by 13% over the controls. However, the damage rating varied significantly across the three cultivars, although there was no significant differences between the treatment levels within each cultivar. The PCR assay successfully amplified 7 of 9 indigenous *B. bassiana* strains including PPRI 7598 and PPRI 7861 at 300bp. Through this study,

endophytic colonisation of five South African wheat cultivars by *B. bassiana* PPRI 7598 was confirmed, presenting new possibilities as IPM component including improved plant growth and potential insect and/or disease suppression.

Keywords: *Beauveria bassiana*, Entomopathogenic fungi, Endophyte, *Triticum aestivum*, *Duraphis noxia*, Inoculation, Pathogenicity, Plant colonisation, South Africa

RESEARCH OUTPUTS

Research articles

1. **L. F. Motholo***, M. Booyse, J. L. Hatting, T. J. Tsilo and O. M. M. Thekiso. (Accepted). Comparison of Wheat growth-response to endophytic *Beauveria bassiana* (Hypocreales: Cordycipitaceae) derived from an insect versus plant host. Australian Journal of Crop Science. Reference No: AJCS-Motholo-PNE1685, In Press.
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ABBREVIATIONS

ARC	Agricultural Research Council
A43	<i>Beauveria bassiana</i> conidiation gene
BCA	Biological control agent(s)
bp	Base pair
CBC	Conservation Biological Control
CTAB	Cetyl trimethyl ammonium bromide
CV	Coefficient of variance
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic acid(s)
DPI	days post inoculation
EPF	Entomopathogenic fungus / fungi
FHB	<i>Fusarium</i> head blight
g/L	Grams per litre
h	Hour(s)
HR	Relative humidity
<i>hyd1 / hyd2</i>	<i>Hydrophobin genes</i>
IPM	Integrated Pest Management

ISR	Induced systemic resistance
ITS	Internal transcribed spacer
KB	Karnal Bunt
LAMP	Loop-mediated isothermal amplification
LC ₅₀	Median lethal concentration from which 50% insect mortality is induced
LSD	Least significant difference
LT ₅₀	Median lethal time within which 50% insect mortality is induced
NWU	North West University
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PPRI	Plant Protection Research Institute
qPCR	Quantitative Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RWA	Russian wheat aphid
RWASA	Russian wheat aphid - South African biotype(s)
SA	South Africa
SAGL	The Southern African Grain laboratory

SANCF	South African National Collection of Fungi
SDA	Sabouraud dextrose agar
SDAY	Sabouraud dextrose agar with 1% yeast extract
SE	Standard error of the mean
USA	United States of America
UV	Ultra-violet light

Scientific units:

cfu	Coliform forming units
ml	Milliliters
mm	Millimeter
°C	Degrees celsius
%	Percentage

CHAPTER 1: INTRODUCTION

1.1 Background

Wheat (*Triticum aestivum* L.) (Poaceae) is the second most important grain produced in South Africa (SA), after maize (Bester, 2014). It contributed 79% of the total winter cereal in the season 2017/18. The wheat production between the dryland and irrigation production in 2017/18 and 2016/17 seasons were comparable. South African wheat production has currently encountered a 16% decline from the average of 1826800 tons to 1535000 tons from the 2007/2008 to 2016/2017 seasons (SAGL, 2018).

Production of wheat is most predominant under dryland conditions in both winter and summer rainfall regions. However, adverse conditions associated with country's climate change are inevitable, of which each 1% decline in rainfall is likely to lead to a 0.5% decline in production of winter wheat and 1.1% decline in maize yield (a summer grain) (Blignaut *et al.*, 2009). Wheat production is also negatively affected by insect pests and diseases which can cause serious damage, compromising profit gained from the crop, hence, producers are either shifting their focus to more profitable commodities or are quitting farming altogether (Bester, 2014).

Owing to their parthenogenetic reproduction and short generation time, aphid populations increase exponentially to rapidly reach pest status and exceed economic threshold levels (Hales *et al.*, 1997). The mechanisms underlying the rapid adaptation in

aphids, is confirmed by polymorphic markers, revealed genetic diversity of aphid populations, and was even evident in parthenogenetic populations (Peccoud and Simon, 2010). Aphid species, causing problems in the winter rainfall area of SA are mainly oat aphid, the English grain aphid and rose grain aphid. Russian wheat aphid (RWA), *Diuraphis noxia* Kurdjumov (Hemiptera: Aphididae), which is the most severe wheat aphid under dryland conditions in South Africa, is a sporadic pest in this area (DAFF, 2016). *Diuraphis noxia* is a widespread and serious pest of cultivated bread wheat (Vandenberg *et al.*, 2001; Tolmay and Van Deventer, 2005) (Fig. 1.1).



Figure 1.1: *Diuraphis noxia* infestation of wheat, *Triticum aestivum* forming colonies of mixed instars. Photograph: Kobus Dreyer, ARC-Small Grain.

Thus, its impact calls for intense management of this notorious insect in agricultural ecosystems. Traditionally, management of *D. noxia* involved application of chemical pesticides, introduction of predators and parasitoids (Hatting, 2002) and development of aphid-resistant cultivars (Souza, 1998).

Through successive chemical applications, aphids developed resistance to several insecticides (Foster *et al.*, 1997; Van Emden and Harrington, 2007). Such response could be ascribed to rapid adaptation to the environment giving rise to large-effect mutations (Messer *et al.*, 2016) or through acquisition of facultative symbionts that confer protection to the host (Henry *et al.*, 2013). In addition to insecticide resistance, host plant resistance has recently become unstable due to the development of resistance-breaking biotypes of *D. noxia* (Tolmay *et al.*, 2006; Jankielsohn, 2014). Unfortunately, aphids have also evolved strong defense mechanisms against parasitoids, following selective pressure exerted by the natural enemy complex (Peccoud and Simon, 2010). However, there is no evidence provided regarding aphid resistance against entomopathogens.

The notion of integrating the EPF *Beauveria bassiana* (Balsamo – Crivelli) Vuillemin (Ascomycota: Hypocreales) with host plant resistance as an alternative IPM approach was explored by Hatting *et al.* (2004) in SA, recording *ca.* 65% fewer aphids on topically treated plants compared to control treatments. No further efforts were however undertaken to investigate the potential of *B. bassiana* alone for control of *D. noxia*. As

a result, the current knowledge gap warrants further efforts for future research based on Hatting's advances to assign native *B. bassiana* isolates for control of *D. noxia* in SA. Secondly, since the previous analysis of endophytes in SA wheat cultivars by Crous *et al.* (1995), no other study has ever analysed the occurrence and impact of fungal endophytes, including *B. bassiana*, against *D. noxia* in SA bread wheat cultivars. In the Crous *et al.* (1995) study, *B. bassiana* was not among the fungal endophytes isolated from SA bread wheat cultivars. The current study investigated the potential use of endophytic *B. bassiana* for control of *D. noxia* on selected SA *T. aestivum* cultivars.

1.2 Problem statement

Aphids (Hemiptera: Aphididae) are destructive sap-sucking pests of agricultural crops. Their exponential increase causes significant damage to both greenhouse and field crops. Species of economic importance, namely, *Myzus persicae* (Sulzer), *Aphis gossypii* (Glover) and *Aulacorthum solani* (Kaltenbach) are common greenhouse crop pests (Down *et al.*, 1996; Sanchez *et al.*, 2007; Van Driesche *et al.*, 2008). Moreover, *Schizaphis graminum* (Rondani), *Rhopalosiphum padi* (Linnaeus) and *D. noxia* can damage wheat, beyond economic threshold levels (Inayatullah *et al.*, 1993; Tolmay, 2006). This knowledge accumulated after *D. noxia* first became widespread in all Ethiopian barley and wheat growing areas by 1976 (Haile, 1981) and was later reported for the first time in 1978 as a wheat pest in SA (Walters, 1984). Chemical insecticides are often used to control aphid populations, but continuous use of such has resulted in resistance

development in many crop-pest systems. This control method is currently discredited due to high chemical costs and toxic residues, affecting the environment and posing risks to human and animal health (Stoate *et al.*, 2001; Berny, 2007; Power, 2010). Therefore, genetic control through the development of resistant cultivars became a valuable option, resulting in the first resistant cultivar to be released in SA in 2002 to reduce frequent use of such chemicals.

However, use of resistant cultivars implies some level of selection pressure, which has led to the development of resistance-breaking aphid biotypes (Tolmay *et al.*, 2006). Evidence of a new resistance-breaking biotype of *D. noxia* was reported in 2003 in Colorado, USA (Haley *et al.*, 2004), while two RWA biotypes were documented on SA wheat in Kenya (Malinga *et al.*, 2007). The phenomenon was recently noted also in SA, where at least four *D. noxia* biotypes are now known (Jankielsohn, 2014). These biotypes have been designated RWASA1 [original entry into SA in 1978], RWASA2 [confirmed in 2005], RWASA3 [2009] and RWASA4 [2011], raising serious concerns for SA wheat production, especially amidst climatic fluctuations (Jankielsohn, 2017).

1.3 Rationale

According to Tolmay and Van Deventer (2005), more than 65% wheat yield loss can be caused by *D. noxia* on susceptible cultivars under dryland conditions in SA. Natural enemy-induced mortality remains an option to reduce the selection pressure enforced

upon aphid populations by resistant cultivars and hence, the development of resistance-breaking biotypes. Therefore, an integrated pest management strategy, in which an entomopathogenic fungus (EPF) such as *B. bassiana* is exploited as endophytic biocontrol agent, has attracted new interest. This initiative will render supportive efforts to address the knowledge gap from the limited work done by Crous *et al.* (1995) on the characterisation endophytes in SA bread wheat cultivars. Moreover, SA's locally maintained isolates of *B. bassiana* have never been explored for their induced systemic resistance (ISR) potential to RWA in wheat. The purpose of this study was to establish and explore indigenous strains of *B. bassiana* as endophyte for ISR in selected SA *T. aestivum* cultivars, thereby acting as additional IPM tool against *D. noxia* infestations.

1.4 Hypothesis

The EPF *B. bassiana*, can be artificially introduced as an endophyte in selected wheat cultivars with resulting systemic resistance against the RWA.

1.5 Aims and objectives

The major aim of this study was to establish the EPF, *B. bassiana*, as an endophyte in wheat, *T. aestivum*, and evaluate its impact on *D. noxia*.

Specific objectives were:

- to conduct laboratory bioassays to select *B. bassiana* isolates highly virulent to Russian wheat aphid, *D. noxia*.
- to evaluate different inoculation techniques for establishing *B. bassiana* as endophyte in *T. aestivum* and measure its impact on seedling growth.

- to evaluate the effect of endophytic *B. bassiana* on the Russian wheat aphid through the measurement of intrinsic rate of increase and other parameters.
- to develop species-specific LAMP assays for detection of *Beauveria bassiana*.

1.6 Expected outcomes

The importance of *B. bassiana* as a plant endophyte for enhanced physiological growth and antagonist against attack by insects and/or pathogens, has become apparent during the past 20 years (Xiao *et al.*, 2012; Vega, 2018). With ISR, crops may benefit more from fungal endophytic activity than from aerial pest control alone. Although some endophyte-related research has been conducted on wheat, in some parts of the world (Gurulingappa *et al.*, 2010; Hassan *et al.*, 2016), no such investigation has to date been considered in SA. Only one study, in which aerially-applied *B. bassiana* was used in combination with host plant resistance against the RWA, was reported by Hatting *et al.*, (2004). The current study proposes development of a candidate strain of *B. bassiana* suitable for endophytic establishment in *T. aestivum* cultivars.

With an anticipated long - term goal, the candidate *B. bassiana* strain will be mass produced and formulated for testing also as endophyte in other SA crops as a commercial mycopesticide and/or plant growth-promoting agent. Such strain will be manipulated for improved conidiation, virulence and resistance to biotic and abiotic stressors. Subsequently, these findings will be applicable for development of efficient

commercial candidate strains with extended shelf life (Mascarín *et al.*, 2016), one of the prerequisite knowledge hurdles that should be addressed before formulation and registration of a commercial product. An additional future prospect is to exploit this candidate strain's potential for control of other sucking arthropods of agricultural economic importance known to vector both veterinary and clinical pathogens.

1.7 Thesis outline

This thesis is structured from a set of specific objectives for this study. All objectives were addressed by accomplishing a set of trials per experiment. Each objective contributed towards a respective experimental chapter (4 chapters) written in the discrete research paper format for publication. Inclusive of literature review and thesis overview, the thesis comprises seven chapters. Chapter 1 brings the general introduction of the study, which consists of the background, problem statement, rationale, hypothesis, the aims and objectives as well as thesis outline. Chapter 2 is the literature review which highlights the ecology of fungal entomopathogens specifically *B. bassiana*, its potential as a generalist pest control agent, its functional role in its association with host plants as an endophyte in pursuit of induced systemic resistance against biotic and abiotic factors and outlines of the general safety of *B. bassiana* as biocontrol agent. Chapters 3, 4, 5, and 6 are experimental chapters, which generated scientific data for publication. The last chapter (Chapter 7) entails the general conclusions and recommendations as an overview of the undertaken research and

suggests future research activities in this field. Each chapter contains references at the end. The referencing system used in thesis chapters is derived from the NWU Harvard referencing style.

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CHAPTER 2: LITERATURE REVIEW

2.1 Background

2.1.1 Ecology of Entomopathogenic fungi (EPF)

The occurrence of EPFs was first studied in the early 19th century (Länge, 2006). Entomopathogenic fungi occur over a wide biogeographical range from forests, deserts, urban regions or orchard soils (Zimmermann, 1986; Chandler *et al.*, 1997). As a result, distribution and diversity of EPFs were studied from different habitats, worldwide (Chandler *et al.*, 1997; Meyling and Eilenberg, 2006; Quesada-Moraga *et al.*, 2007; Shin *et al.*, 2013). The association of these fungi with soil mainly benefits these microbes with protection against biotic and abiotic factors, which can affect EPF distribution, particularly UV light (Keller and Zimmermann, 1989). Apart from soil, other environmental factors such as climate, geographic location, habitat and cropping system also affect EPFs distribution (Khudhair *et al.*, 2014).

EPFs are considered important regulators of insect populations in terrestrial ecosystems (Hajek, 1997), natural enemies (Roy *et al.*, 2009) and traditional mortality factors for insect pests in agricultural and natural ecosystems (Meyling and Eilenberg, 2007; Vega *et al.*, 2009; Sánchez-Peña *et al.*, 2011). Recently several studies have been directed at the development of EPFs as biological agents against common pests within agroecosystems (Meyling and Eilenberg, 2007). For the same notion, Lockwood (1993) discourages the habitual use of imported isolates to control pests since EPFs efficacy is

environmentally dependent. This suggests that interactions between the host, fungus and the environment are diverse and dynamic hence EPFs occupy a wide range of habitats (Chandler, 2009). Therefore, it is important to inspect local agroecosystems biodiversity of EPFs for selection as reliable biological control agents (BCA) (Khudhair *et al.*, 2014). However, very little has been done on understanding the fundamental ecology of EPFs in agroecosystems (Meyling and Eilenberg, 2007). Subsequently, little is known about the factors that influence their distribution, population structure, econutritional behaviour and the evolution of virulence related characteristics (Chandler, 2009). Some EPF genera such as *Beauveria*, *Metarhizium*, *Paecilomyces* and *Isaria* usually inhabit soil for some part of their life cycle as saprophytic organisms (Domsch *et al.*, 1980; Keller and Zimmerman, 1989; Toledo *et al.*, 2008). Knowledge of their occurrence and distribution helps to connect them to their geographical locations and facilitate a better indication of the possible biological agent to use against pests (Quesada-Moraga *et al.*, 2007). According to Längle (2006) and Zimmerman (2007), the EPF genus *Beauveria* among the EPF complex was first studied in the 19th Century introducing its two common species, *Beauveria bassiana* (Balsamo – Crivelli) Vuillemin (Ascomycota: Hypocreales) and *B. brongniartii* (Saccardo) Petch. Other *Beauveria* species were recently identified in different studies (Table 2.1).

Table 2.1: Representation of documented species of *Beauveria* genera as described from different studies*

Species	Host /substrate	Conidia size (µm)	Conidia shape	Reference
<i>Beauveria bassiana</i> (Bals.-Criv.) Vuill	Hymenoptera, Coleoptera, Lepidoptera, Hemiptera; soil	Variable	Globose/ Ellipsoidal	Vuillemin (1912); Rehner and Buckley (2005), Rehner <i>et al.</i> 2011); Cheng <i>et al.</i> 2013; Robène-Soustrade <i>et al.</i> (2015)
<i>B. brongniartii</i> (Sacc.) Petch	Coleoptera, Homoptera, Lepidoptera	Variable	Ellipsoidal	Petch (1926)
<i>B. vermiconia</i>	Soil	2.5-3.5 x 2.5-3	Comma shape	de Hoog and Rao (1975)
<i>B. caledonica</i>	Coleoptera, Orthoptera, soil	variable	Ellipsoidal/ cylindrical	Bisset and Widde (1986)
<i>B. malawiensis</i>	Coleoptera, soil	3.7-5.5 x 1.3-3	Cylindrical	Rehner <i>et al.</i> (2006); Rehner <i>et al.</i> (2011); Chen <i>et al.</i> (2013); Chen <i>et al.</i> (2013); Robène-Soustrade <i>et al.</i> (2015)
<i>B. amorpha</i> (Höhn.) Minnis	Hymenoptera, Coleoptera, soil	3.5-5 x 1-2	Cylindrical	Samson and Evans (1982); Rehner <i>et al.</i> (2011); Chen <i>et al.</i> (2013), Chen <i>et al.</i> (2013); Robène-Soustrade <i>et al.</i> (2015)
<i>B. asiatica</i>	Coleoptera	4-5 x 2-3	N/A	Rehner <i>et al.</i> (2011); Robène-Soustrade <i>et al.</i> (2015)
<i>B. australis</i>	Orthoptera, soil	N/A	N/A	Rehner <i>et al.</i> (2011)
<i>B. kipukae</i>	Homoptera	N/A	N/A	Rehner <i>et al.</i> (2011)
<i>B. pseudobassiana</i>	Hymenoptera, Coleoptera, Lepidoptera, Hemiptera, Thysanoptera	3-4 x 2.5-3.5	Globose/ Ellipsoidal	Rehner <i>et al.</i> (2011); Robène-Soustrade <i>et al.</i> (2015)
<i>B. sungii</i>	Coleoptera	4.5-6 x 2.5-3.5	Ellipsoidal	Rehner <i>et al.</i> (2011); Robène-Soustrade <i>et al.</i> (2015)
<i>B. varroae</i>	Coleoptera	N/A	N/A	Rehner <i>et al.</i> (2011)
<i>B. lii</i>	#N/A	3.1-10.1 x 1.4-3.6	Ellipsoidal	Zhang <i>et al.</i> 2012; Chen <i>et al.</i> , 2013
<i>B. sinensis</i>	Lepidoptera	3-5 x 1.5-2	Ellipsoidal/ cylindrical	Chen <i>et al.</i> (2013)
<i>B. hoplocheli</i>	Coleoptera	Variable	Cylindrical	Robène-Soustrade <i>et al.</i> (2015)
<i>B. majiangensis</i>	Coleoptera	3.8-12.9 x 1.2-1.5	Cylindrical	Chen <i>et al.</i> (2018)
<i>B. medogensis</i>	N/A	N/A	N/A	Chen <i>et al.</i> (2018)
<i>B. araneola</i>	N/A	N/A	N/A	Chen <i>et al.</i> (2018)

* Adapted from Rehner and Buckley (2005); Rehner *et al.* (2011); Chen *et al.* (2013); Robène-Soustrade *et al.* (2015)

*2.1.2. Ecology of entomopathogenic fungus *Beauveria bassiana* in Agriculture*

Beauveria bassiana is a widely known EPF. The occurrence of this fungus in different soil habitats was documented globally, including SA (Table 2.2).

Table 2.2: Natural occurrence and distribution of *Beauveria bassiana* in different soil types from various countries. Adapted from Zimmermann (2007).

Location	Soil habitat	Isolated EPF species	Reference
Canada	Different soil samples	<i>Beauveria bassiana</i> and <i>Metarhizium anisopliae</i>	Bidochka <i>et al.</i> (1980)
Czech Republic	Different soil types including soils from arable conventional and organic farms	<i>B. bassiana</i>	Landa <i>et al.</i> (2002)
Finland	Different soil types	<i>B. bassiana</i>	Vanninen (1996)
Germany	Different soil types	<i>B. bassiana</i>	Kleespies <i>et al.</i> (1989)
Italy	Different soil types	<i>B. bassiana</i>	Tarasco <i>et al.</i> (1997)
Japan	Forest soils	<i>B. bassiana</i>	Shimuza <i>et al.</i> (2002)
Macquarie Islands	Subantartic soils	<i>B. bassiana</i>	Roddam and Rath (1997)
Nepal	Different soil types	<i>B. bassiana</i>	Dhoj and Keller (2003)
New Zealand	Pasture, forest and cropland	<i>B. bassiana</i> (most abundant in pasture soils than forest or cropland soils)	Barker and Barker (1998)
Norway	Soils from arable organic and conventional farms.	<i>B. bassiana</i> , <i>M. anisopliae</i> , and <i>Tolypocladium cylindrosporum</i> (most abundant on organic farms)	Klingen <i>et al.</i> (2002)
Panama	Tropical forest soils near colonies of leaf-cutting ants	<i>B. bassiana</i>	Hughes <i>et al.</i> (2004)
Poland	Apple and plum orchards (soils under sward)	<i>B. bassiana</i>	Sapieha-Waszkiewicz <i>et al.</i> (2003)
	Hop plantations and arable fields	<i>B. bassiana</i>	Mietkiewski <i>et al.</i> (1996)
	Mid-forest meadows	<i>B. bassiana</i> , <i>B. brongniartii</i>	Mietkiewski <i>et al.</i> (1994)
	Different soil types	<i>B. bassiana</i>	Tkaczuk and Mietkiewski (1996)
Spain	Different soil types	<i>B. bassiana</i>	Asensio <i>et al.</i> (2003)
Switzerland	Soil sites colonised by <i>Melolontha melolontha</i>	<i>B. brongniartii</i> and <i>B. bassiana</i>	Keller <i>et al.</i> (2003)
USA	Soils from orchards	<i>B. bassiana</i> and <i>M. anisopliae</i>	Shapiro-Ilan <i>et al.</i> (2003)
South Africa:			
Eastern Cape	Different soil types	<i>B. bassiana</i>	Goble (2009); Coombes (2012)
North West	Different soil types	<i>B. bassiana</i>	Morar-Bhana <i>et al.</i> (2011)
Western Cape	Different soil types	<i>B. pseudo-bassiana</i>	Abaajeh <i>et al.</i> (2014)

Additional investigations demonstrated that *B. bassiana* was found to be the most widely distributed species of this genus. As such, much attention has been recently directed at developing this EPF as biological control agent (BCA) against common forestry and agricultural pests (Meyling and Eilenberg, 2007) including aphids. The most important attribute reported with this EPF is its ability to associate with plants as a symbiont and endophyte as confirmed in various crops in many studies (Vega, 2008, 2018).

2.2 Topical application of *B. bassiana* against aphids

Topical application of entomopathogens for aphid control is applicable either under laboratory/glasshouse conditions using different equipment. Based on individual requirements, hand-held atomisers, knap sack sprayers or Burgerjon spray towers can be used in the laboratory, whereas computer-controlled sprayers and tractor mounted devices (Hatting and Wraight, 2007) or overhead irrigation systems are suitable use for use in the fields (Vandenberg *et al.*, 2001). Spray inocula for control of aphids and other insect pests are generally formulated from different EPF viable conidia (Faria and Wraight, 2007). Most EPFs including *B. veria bassiana* strains were globally formulated and commercialised for topical application against different aphid species (Inglis *et al.* 2001; Maina *et al.*, 2018). Currently about 71 mycoinsecticides and mycoacaricide products are registered worldwide for pest control, with 58 products formulated from *B. bassiana* (Faria and Wraight, 2007). Mycopesticide products formulated from *B.*

bassiana range from liquid emulsion to powdered products grown in different growth media (Glare, 2004).

In SA commercial products such as Bb weevil[®], ECo Bb[®] and Bb Plus[®] formulated from *B. bassiana* strains are available for control of certain Hemiptera (Aphididae), Acari (Tetranychidae) and Coleoptera (Curculionidae) species (Faria and Wraight, 2007). However, there is no *B. bassiana* product currently available against *D. noxia*. In a study conducted in SA, topical application (Fig. 2.1) with *B. bassiana* strain GHA (exotic commercial strain), using a Burgerjon spray tower reduced *D. noxia* population by 65% in wheat compared to the control (Hatting *et al.*, 2004).

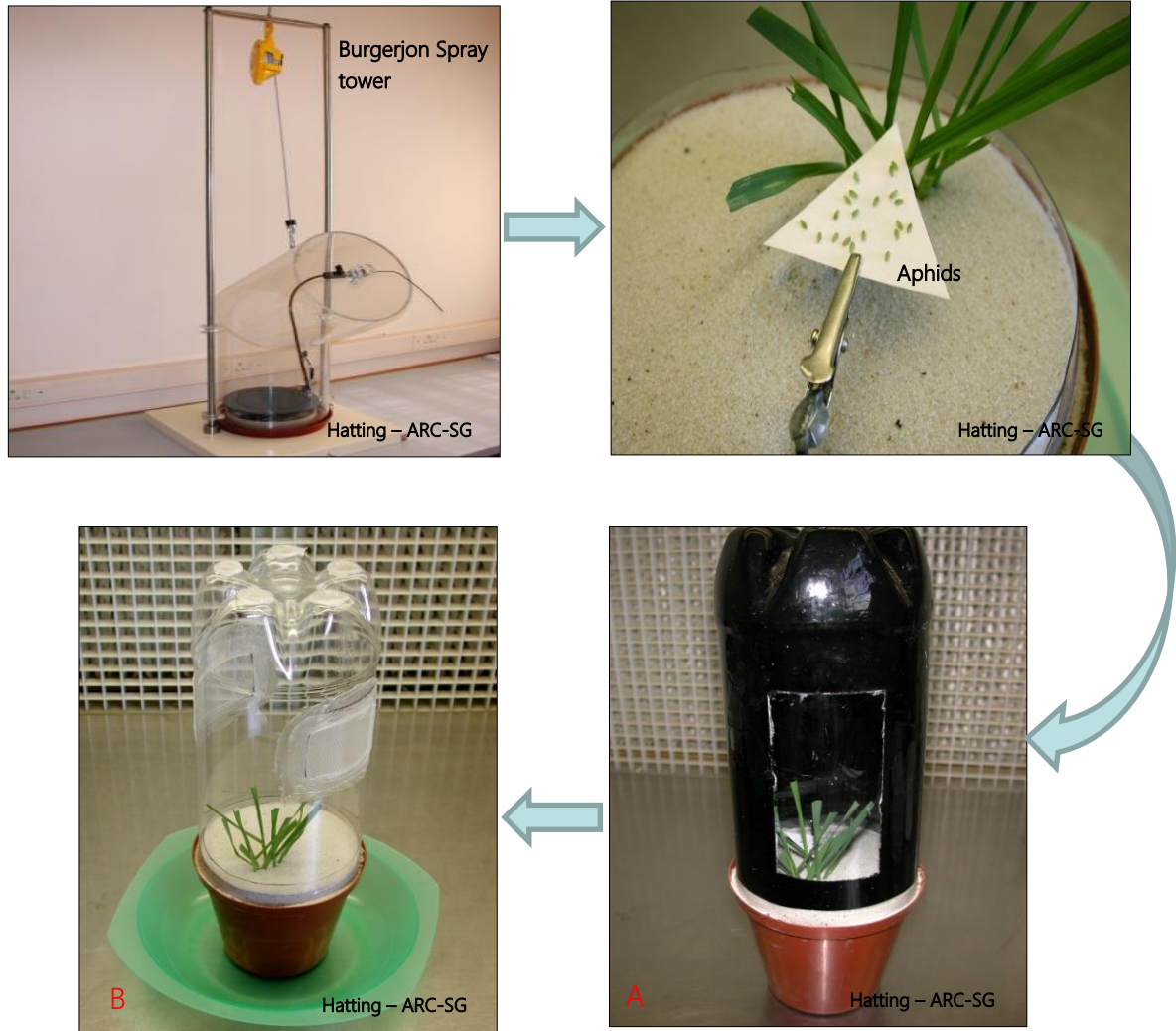


Figure 2.1: Topical application with *B. bassiana* conidial suspension on the Russian wheat aphid, *D. noxia* using the Burgerjon spray tower in the laboratory. **(A)** Aphids are maintained on wheat seedlings in black cages to provide a full darkness incubation for 24 hours post inoculation under glasshouse conditions. **(B)** Aphids incubated in clear cages also maintained in the glasshouse under natural light for 6 days post inoculation (DPI). Incubation structures adapted from Hatting and Wraight (2007).

In agreement with these results, during the study by Gurulingappa *et al.* (2011), topical application with *B. bassiana* and *Lecanicillium attenuatum* (Zimmermann) Zare and Gams against aphids had a negative impact on *Aphis gossypii*.

When applied under controlled environmental conditions in the laboratory or glasshouse, *B. bassiana* strains induced high aphid mortality at varying concentration regimes of 10^6 , 10^7 and 10^8 conidia ml^{-1} within 5-7 days after inoculation (Hatting and Wraight, 2007). Alternatively, an equivalent of 10^{13} conidia ha^{-1} under field conditions caused mycosis among *D. noxia* within 5 days of inoculation on irrigated spring and winter wheat (Vandenberg *et al.*, 2001). Interestingly, the same concentration caused mortality within 4-5 weeks of application under rain fed systems (Filho *et al.*, (2011), suggesting the effect of humidity or moisture on the efficacy of EPF towards insect mortality and development of mycosis. In another study, the 50% lethal concentration (LC_{50}) values of the inoculum were determined in conjunction with the 50% lethal time (LT_{50}) values on *Myzus persicae* (Sulzer) (Rhynchotha: Aphididae) which decreased with an increasing conidial concentration ml^{-1} (Vu *et al.* 2007).

In SA, LC_{50} values of commercial *B. bassiana* isolate (GHA) against aphids and whiteflies on wheat were estimated in a laboratory assay optimisation procedure (Hatting and Wraight, 2007). In this study, seedlings with resident test aphids were maintained under glasshouse conditions (25 ± 2 °C, $40 \pm 5\%$ HR under natural light). Higher concentration of 10^8 caused the highest LC_{50} against aphids.

2.2.1 Infection process of Beauveria bassiana in insect hosts

The infection process of the insect host forms part of the life cycle of entomopathogens (Zimmermann, 2007; Fig. 2.2). Susceptibility of insect hosts to EPFs is explicitly based on interactions between conidia and the insect epicuticle, the outermost layer and the first point of contact between the pathogen and the host. The infection starts from dispersal and transmission of fungal propagules for attachment on the epicuticle surface. This interaction defines the success of the fungus to cause mycosis or the host to clear the fungus. The infection process consists of conidia attachment to the cuticle (adhesion), germination and penetration, overcoming of the host response and immune responses, proliferation and formation of hyphal bodies (blastospores) and saprophytic outgrowth from the dead host (Zimmermann, 2007).

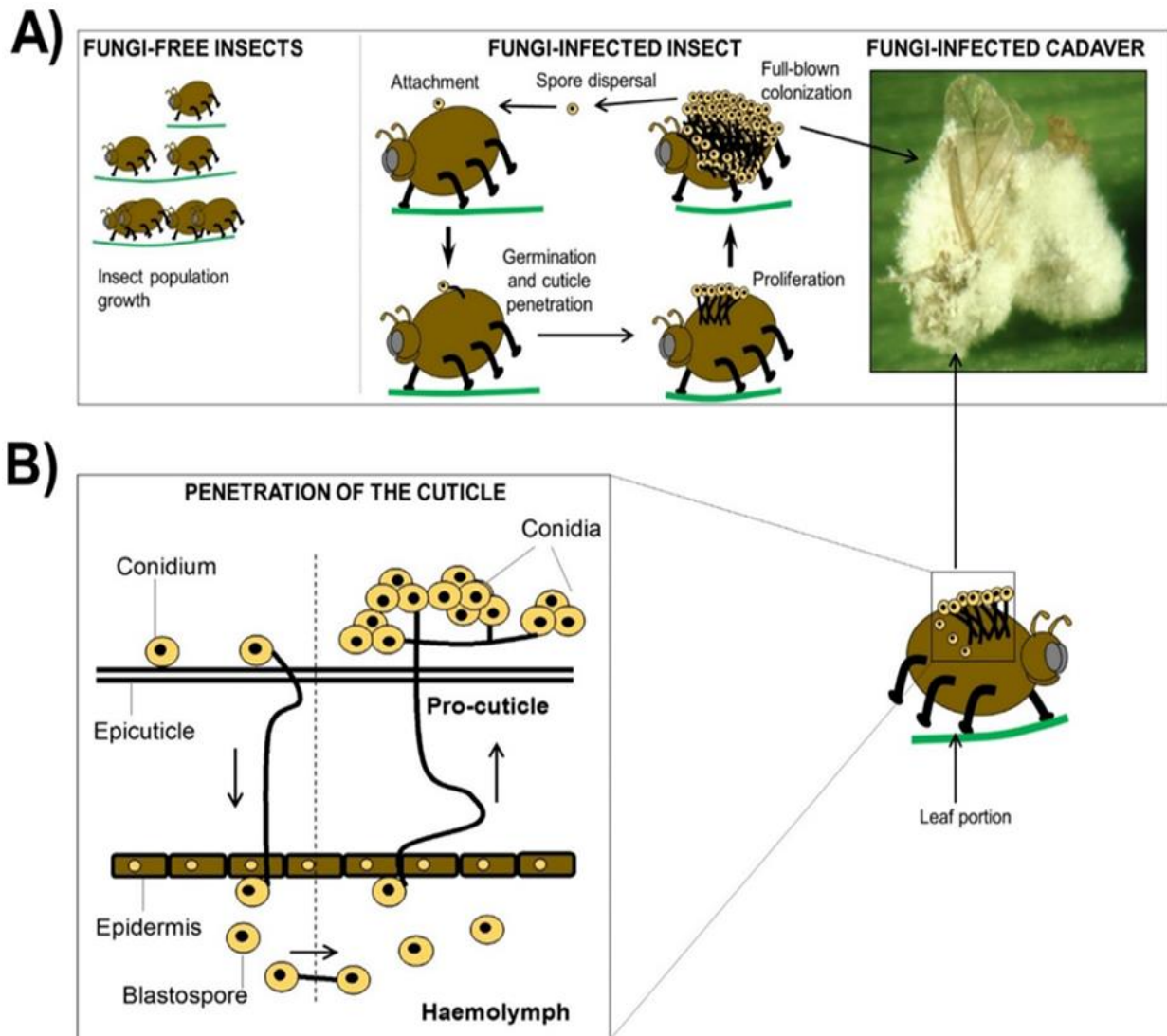


Figure 2.2: The Schematic diagram of entomopathogenic fungal infection process in insects: **(A)** Fungal infection on the host; saprophytic outgrowth and sporulation of *Beauveria bassiana* on the insect cadaver. **(B)** Graphic presentation of fungal entomogenous infection process through the insect cuticle. Drawn with adaptation from Clarkson and Charnley (1996). Fungi-infected aphid cadaver photograph: Dr J.L Hatting, ARC- Small Grain.

Adhesion: In brief, during the initial stage of infection, single-celled fungal conidia attach to the insect cuticle. Conidial adhesion of *B. bassiana* is facilitated by two hydrophobin genes, *hyd1* and *hyd2* (Zhang *et al.*, 2011). Hydrophobins are responsible for binding *B. bassiana* conidia to the hydrophobic surfaces of the substrate and subsequently activate fungal virulence. On the other hand, hydrolytic enzymes such as

proteases, chitinases and lipases, are secreted to promote germination and fungal growth on, and penetration of the insect cuticle (Ortiz-Urquiza and Keyhani, 2013).

Penetration: Successful conidial adherence to the insect cuticle augments conidial germination through synergist activity of conidial surface proteins capable of cuticle degradation (Ortiz-Urquiza and Keyhani, 2013). Penetration through the intercellular spaces between cuticular cells into the hemocoel of the insect host is primarily facilitated through mechanical pressure and enzymatic degradation. During this process the fungus develops specialised infection structures called appressoria, which promote hyphal penetration into the cuticle (Holder *et al.*, 2007). Formation of this structure results from expression of perilipin (*Mpl1*) in EPFs, which enhances breakdown of endogenous lipids and increases turgor pressure required for cuticular penetration. For this notion, appressoria are substantially implicated for successful penetration of the cuticle for the enhanced virulence (Barelli *et al.*, 2016). For example, *M. brunneum* can potentially express various proteases on the host cuticle in different stages of pathogenesis, which presumably engage *Metarhizium* strains to adapt to different habitats (Barelli *et al.*, 2016). This implies the role of proteases in virulence and host recognition irrespective of the habitat.

Proliferation: Similar to *M. brunneum*, once in the hemocoel, *B. bassiana* forms structures typically formed by pathogenic yeasts (e.g. *Candida albicans*) called blastospores. Both *B. bassiana* and *M. brunneum* produce toxins or secondary

metabolites (beauvericin and destruxins,) which allow blastospores to proliferate within the haemolymph by absorbing and depleting nutrients, finally killing the insect host within 3-7 days (Zimmermann, 2007). In the end, the fungal hyphae proliferate throughout the insect cadaver and emerge to form conidia on the mummified host (Schrank and Vainstein, 2010). *Beauveria* infections are characterised by symbolic white conidia, while *Metarhizium* produces green conidia on the surface of the infected cadaver. Therefore, fungal infections on the hosts remains the only part of the fungal life cycle where there can be a build-up of significant population sizes through production of conidia (Meyling and Eilenberg, 2007).

2.3 Endophytic associations with plants

In natural agroecosystems, plants are commonly associated with symbiotic microorganisms such as bacteria and fungi existing as endophytes. The term endophyte was first introduced by the German scientist Heinrich Anton de Barry in 1866, Carroll (1986) and was used to define bacteria and fungi which colonise the internal tissue of healthy plants without causing any apparent disease symptoms in their host plants (Wilson, 1995; Schulz and Boyle, 2005). Endophytes are widespread and diverse in both natural and agricultural ecosystems and can colonise almost all plant parts (Stone *et al.*, 2000). Over a long period of evolution, plants and co-existing endophytes have established a special association, which increases their adaptability and tolerance to biotic and abiotic stresses in the environment (Jia *et al.*, 2016). Notably, some

endophytes have no apparent effects on plant performance but live on host plant metabolites. These are termed commensal endophytes, whereas other endophytes like *Fusarium verticillioides* confer beneficial effects to their host plant as they suppress other pathogenic fungi (e.g. *Ustilago maydis*) (Scortichini and Loreti, 2007; Estrada *et al.*, 2012).

Fungal endophytes are the most studied group of endophytes, most of which have been detected in various higher plants (Azevedo *et al.*, 2000) and agricultural commodities like wheat (Larran *et al.*, 2002a; Gurulingappa *et al.* 2010); bananas (Pocasangre *et al.*, 2000; Cao *et al.*, 2002); soybeans (Larran *et al.*, 2002b); tomatoes (Larran *et al.*, 2001) (Table 2.3). Endophytes naturally colonise plants through different inocula dispersal mechanisms in the environment, or when artificially inoculated to plants (Russo *et al.*, 2015; Sánchez-Rodríguez *et al.*, 2015; Sánchez-Rodríguez *et al.*, 2018).

Table 2.3: A list of plants colonised endophytically by the EPF species *Beauveria bassiana*, *Metarhizium anisopliae* and *Lecanicillium lecanii*

Fungal species	Plant	Reference
<i>Beauveria bassiana</i>	<i>Zea mays</i> L. (Poaceae) maize	Vakili (1990); Bing and Lewis (1991; 1992a, b; Jones, 1994; Lomer <i>et al.</i> , 1997; Cherry <i>et al.</i> , 1999, 2004; Wagner and Lewis, 2000; Lewis <i>et al.</i> , 2001)
	<i>Z. mays</i> (L)	Jones (1994); Arnold and Lewis (2005)
	<i>Solanum tuberosum</i> L. (Solanaceae) Potato,	Jones (1994)
	<i>Gossypium hirsutum</i> L. (Malvaceae) Cotton	Jones (1994)
	<i>Lycopersicon esculentum</i> Miller (l) (Solanaceae) Garden tomato	Leckie (2002); Ownley <i>et al.</i> (2004)
	<i>Theobroma gileri</i> Coatrec; <i>Theobroma cacao</i> L. (Malvaceae) Cocoa	Evans <i>et al.</i> , (2003); Posada and Vega (2005)
	<i>Carpinus caroliniana</i> Walter (Betulaceae) Bark of ironwood	Bills and Polishook (1991)
	<i>Pinus monticola</i> D. Don N. (Pinaceae) Western white pine	Ganley and Newcombe (2005)
	<i>Papaver somniferum</i> L. (Papaveraceae) Opium poppy	Quesada-Moraga <i>et al.</i> (2006)
	<i>Phoenix dactylifera</i> L. (Arecaceae) Date palm	Gómez-Vidal <i>et al.</i> (2006)
	<i>Musa paradisiaca</i> L. (Musaceae) Banana	Akello <i>et al.</i> (2007)
	<i>Coffea arabica</i> L. (Rubiaceae) Coffee	Posada and Vega (2006); Posada <i>et al.</i> (2007); Vega <i>et al.</i> (2008, 2010)
	<i>Corchorus olitorius</i> L. (Malvaceae) Jute	Biswas <i>et al.</i> (2012)
	<i>Phaseolus vulgaris</i> (Leguminosae) Common bean	Parsa <i>et al.</i> (2013)
	<i>Cynara scolymus</i> L. (Asteraceae) Artichoke	Guesmi-Jouini <i>et al.</i> (2014)
	<i>Cucurbita pepo</i> L. (Cucurbitaceae) Squash	Jaber and Salem (2014)
	<i>Vitis vinifera</i> L. (Vitaceae) Grapevine	Jaber (2015)
	<i>Brassica napus</i> L. (Brassicaceae) Oilseed rape	Vidal and Jaber (2015)
	<i>Vicia faba</i> L. (Fabaceae) Broad bean	Jaber and Enkerli (2016)
	<i>Nicotiana tabacum</i> L. (Solanaceae) Tobacco; <i>Glycine max</i> (L.) Merr. (Fabaceae) Soybean	Russo <i>et al.</i> (2015)
	<i>Triticum aestivum</i> L. (Poaceae) Wheat	Gurulingappa <i>et al.</i> (2010); Russo <i>et al.</i> (2015); Hassan <i>et al.</i> (2016)
	<i>Solanum lycopersicum</i> L. (Solanaceae) Tomato	Hassan <i>et al.</i> (2016)
<i>Metarhizium anisopliae</i>	<i>Glycine max</i> L. (Fabaceae) Soybean	Khan <i>et al.</i> (2012)
	<i>Quercus robur</i> (Fagaceae) English oak	Kwasna <i>et al.</i> (2016)
	<i>Taxys chinensis</i> (Taxaceae) Chinese yew	Liu <i>et al.</i> (2009)
	<i>Solanum lycopersicum</i> L. (Solanaceae) Tomato	Garcia <i>et al.</i> (2011)
	<i>Vicia faba</i> L. (Fabaceae) Broad bean	Akello and Sikora (2012)
<i>Lecanicillium lecanii</i> (current name = <i>Akanthomyces lecanii</i>)	<i>Manihot esculenta</i> (Euphorbiaceae) Cassava	Greenfield <i>et al.</i> (2016)
	<i>Dactylis glomerata</i> (Poaceae) Orchard grass	Sanchez -Marquez <i>et al.</i> (2007)

Endophytic colonisation of plants is characterised by induced systemic resistance (ISR) mechanism in host plants and subsequently protects plant parts against future attack by pathogenic microbes (Dingle and McGee, 2003; O'Hanlon *et al.*, 2012) and herbivorous insects (Azevedo *et al.*, 2000; Van Wees *et al.*, 2008; Segarra *et al.*, 2009; Hossain *et al.*, 2016). In the recent review, Vega (2018) confirms the use of EPFs as endophytes being the novel alternative for insect pests and plant disease management. Additionally, fungal endophytes may protect plants against plant parasitic nematodes (West *et al.*, 1988; Waweru *et al.*, 2014). On the other hand, fungal endophytes may confer beneficial relationships as they interact with their host plants after colonisation (Jia *et al.*, 2016). As highlighted by Firakova *et al.* (2007) and Rodriguez *et al.* (2009), endophytes produce different bioactive compounds (such as alkaloids, diterpenes, flavonoids and isoflavonoids), which enhance biotic and abiotic stress resistance to their host plants. They also involve production of different plant hormones that enhance plant growth in their host plants (Waqas *et al.*, 2012). For example, *Azospirillum* species may enhance wheat (*Triticum aestivum* L) growth under drought stresses (Dingle and McGee, 2003). Additionally, some endophytic fungi such as *Piriformospora indica* also promoted host plant (tobacco) growth by activating the expression of some essential genes and enzymes (Chen *et al.*, 2005) such as nitrate reductase and glucan-water dikinase (starch – degrading enzyme) (Sherameti *et al.*, 2005).

2.3.1 Endophytic functional plant responses

2.3.1.1 Growth Promotion

In recent studies, the well known hypocrealean endophytes, *B. bassiana* and *M. anisopliae* were evaluated for endophytic colonisation and were confirmed to be plant growth promoters in different crops (Akello *et al.*, 2008; Dara, 2013; Jaber and Enkerli, 2016; Sánchez-Rodríguez *et al.*, 2018).

Of many growth parameters, endophytes improve plant fitness and biomass by activating the expression of some essential enzymes (Chen *et al.*, 2005) such as nitrate reductase and glucan-water dikinase (starch – degrading enzyme) (Sherameti *et al.*, 2005). Endophytes may acquire essential plant nutrient elements such as nitrogen and phosphorus from the environment (Zhang *et al.*, 2006; Hartley and Gange, 2009).

Naturally, endophytes engage different mechanisms to acquire these nutrients. Bacterial endophytes may use nitrogen fixation mechanisms (Jha and Kumar, 2007), phosphate solubilisation (Verma *et al.*, 2001; Wakelin *et al.*, 2004) and iron sequestration to alleviate iron chlorosis symptoms in plants (Sánchez-Rodríguez *et al.*, 2015; Sánchez-Rodríguez *et al.*, 2016). Endophytic bacteria may potentially produce indole acetic acid (Leew *et al.*, 2004) and siderophore (Costa and Loper, 1994) to promote plant growth (Chaturvedi *et al.* 2016) (Fig. 2.3) and enhance host plant fitness and increase plant biomass (Rodríguez *et al.*, 2009).

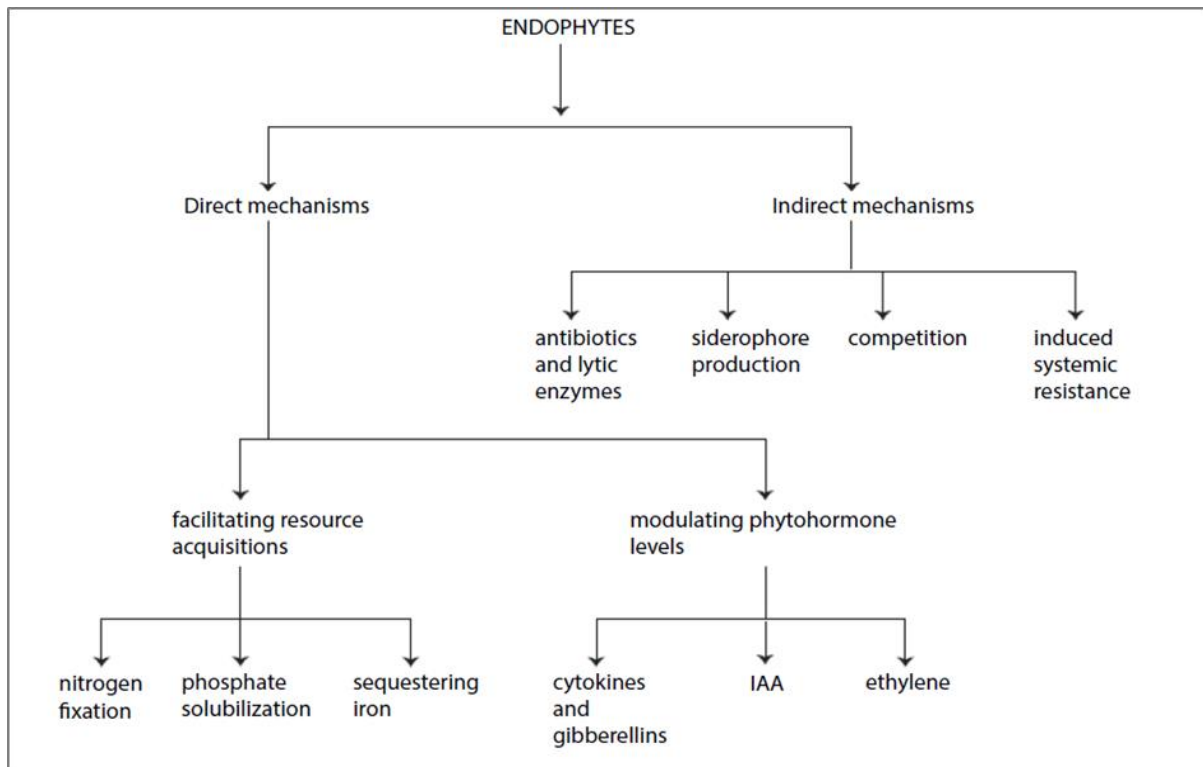


Figure 2.3: Outline of various mechanisms adapted by endophytes to promote plant growth. Adapted from Chaturvedi *et al.* (2016).

Interestingly, recent reports indicated that *Metarhizium robertsii* translocated nitrogen directly from mycosed insect cadavers to plants through hyphae and enhanced plant growth (Behie *et al.*, 2012). Additional to enhanced mineral uptake and alterations of nitrogen accumulation for enhanced metabolism, fungal endophytes also portray other beneficial benefits on plant growth, notably, osmotic adjustment, stomatal regulation and root morphological modification (Compant *et al.*, 2005).

Enhancement of nutrient uptake through endophytic activities is the most crucial attribute to plant growth and improved plant health, hence such evidence advocates endophytes as potential plant growth promoters.

2.3.1.2 Insect suppression

Fungal endophytes are perceived to be mutualists as they potentially receive nutrition and protection from their host plants. In this mutual association, endophytes confer resistance to their host plants against insect herbivory (Clay, 1990) by producing alkaloid-based compounds toxic to certain insect species (Faeth, 2002) thus improving plant defence against insect attack. Subsequently, endophytic fungi also indirectly benefit their host plants by altering plant nutritional contents and morphological traits of insect herbivores thereby creating competition between the fungus and the insect herbivore for limited nutritional resources from the host plant (Hendrix, 1970). Increased amounts of manganese found in *B. bassiana* inoculated plants marked an enhanced protection against insect herbivore following host plant endophytic colonisation (Sánchez-Rodríguez *et al.*, 2018). This evidence confirms the findings by Gurulingappa *et al.* (2010) that the presence of *B. bassiana* and *Lecanicillium lecanii* (Zimm.) Zare and Gams decreased aphid population growth in crops including wheat, while consumption of *B. bassiana* and *Aspergillus parasiticus* Spear colonised wheat leaves also slowed locust nymphs growth.

2.3.1.2 Plant pathogen suppression

The roles of fungal endophytes are ascribed to shaping of plant - environment interactions by protecting plants against plant pathogens (Dingle and McGee, 2003; O'Hanlon *et al.*, 2012). They also secrete some lytic enzymes (antibiotics) that constitute

antifungal, antibacterial and insecticidal activities that potentially inhibit growth of other microorganisms (Gunatilaka, 2006). Endophytic *B. bassiana* and *Lecanicillium* spp. can potentially exhibit antagonism against plant pathogens in different crops (Jaber and Ownley, 2018). This evidence was presented when *B. bassiana* reduced the severity of bacterial blight in cotton seedlings (Ownley *et al.*, 2008), Zucchini yellow mosaic virus in squash (Jaber and Salem, 2014), and downy mildew in grapevines (Jaber, 2015).

Based on the report by Gothandapani *et al.* (2015), *B. bassiana* and *M. anisopliae* possess pathogen inhibitory effects, notably, antifungal activity of *B. bassiana* on *Botrytis cinerea* and *Fusarium oxysporum*. Further reports suggest that treatment of seeds with *Clonostachys rosea* combined with *M. brunneum* inoculum reduced *Fusarium culmorum* infections in wheat (Keyser *et al.*, 2016). Generally, the stated findings confirm the potential of endophytes to modify plant disease occurrence in different crops (Busby *et al.*, 2016).

2.3.1.3 Nutrient exchange enhancers

Soil always remains the crucial nutrient source for plants in both agricultural and natural ecosystems. This resource becomes a limiting factor for plant growth particularly when it releases insufficient nutrient supply to plants. Conversely, plants overcome nutrient shortage as they adapt to symbiotic mutual associations with some soil inhabiting bacteria, fungal mycorrhiza and fungal endophytes (Clark and Zeto, 2000). As indicated by Govindarajulu *et al.* (2005); Guether *et al.* (2009); Behie and Bidochka (2014)

pertaining to the fungal-plant association, fungal symbionts potentially transfer limited nitrogen and phosphorus to their host plants in exchange for plant-derived carbohydrates (Bonfante and Genre, 2010). Subsequently, plants colonised by fungal endophytes benefit through access to the specialised nitrogen reservoir in the soil where they can also reacquire nitrogen lost through insect herbivory (Barelli *et al.*, 2016).

2.3.1.4 *Bioactive compounds and secondary metabolite producers*

Most endophytic microbes isolated from plants were tested for their capacity to produce secondary metabolites same as their host plants (Begum and Tamilselvi, 2016). Some endophytic fungi could produce different classes of phytochemicals or secondary metabolites original from medicinal plants. Some exhibited the ability to promote accumulation of secondary metabolites of host plants that could improve the quality and quantity of drugs (Chen *et al.*, 2016). In addition, other endophytic fungi promote formation of secondary metabolites that could only be produced by host plants. Notably, the endophyte *Coetotrichum gloesporioides* could induce production of artemisinin in *Artemisia annua* (Wang *et al.*, 2006), a common compound that functions as a bioactive compound for antitumor, antimalarial, antipyretic, analgesic or anti-inflammatory activities for medicinal therapies. In subsequence, their capacity to produce secondary metabolites warrants them the potential to release some important plant growth hormones such as auxins and cytokinins (Dalal *et al.*, 2013).

2.3.1.5 *Host plant stress tolerance enhancers*

Enhanced tolerance to multiple abiotic stressors has been observed within endophytic - infected grass species (Kuldau and Bacon, 2008). Hirt (2009) commented that all plants perceive and respond to stress signals of abiotic (drought, heat, salinity) and biotic (herbivores and pathogens) stressors. Responsively, production of fungal secondary metabolites (ergot and loline alkaloids) in endophytic-infected grasses was implicated as the common drought tolerance mechanism that regulates the osmotic potential of plants to reduce the effects of drought stress (Bush *et al.*, 1997). Loline alkaloids are also known to display a dual role in grass protection from biotic (insect pests) and abiotic (drought) stresses (Bush *et al.*, 1997).

Plant tolerance to biotic stresses is correlated with fungal endophytic production of bioactive compounds that have antimicrobial activity against pathogens (Gunatilaka, 2006). Fungal endophytic-plant induced stress tolerance could warrant such plants capability to outcompete non-infected plants and consequently become invasive to control or native plants (Clement *et al.*, 2005). On the other hand, endophytic fungal symbiosis may stimulate biosynthesis of plant hormones and flavonoids that can lower production of phytoestrogen or isoflavone (daidzein and genistein) contents in soybean under salinity stress (Khan *et al.*, 2012). Additionally, the fungal endophyte *Fusarium culmorum* also confers salt tolerance to plants as a complementary activity against salinity stress (Khan *et al.*, 2012).

According to Sgobba *et al.* (2015), heat stress is also one of the most recently experienced abiotic stresses amidst increasing temperatures (Jankielsohn, 2016). Heat stress interrupts plant growth by impairing essential plant physiological and morphological processes such as seed germination, growth rate, plant height, photosynthesis, flowering and subsequent premature cell death (Hasanuzzaman *et al.*, 2013; Sgobba *et al.*, 2015). However, depending on habitat specificity, plant symbiosis with some endophytic fungi such as *Curvularia protuberata* may confer heat tolerance traits preventing photobleaching of plants exposed to heat stress (Rodriguez *et al.*, 2008).

2.3.2 Endophytes associated with the family Poaceae

Based on the findings by Vidal and Jaber (2015), specialist endophytic fungal species include Clavicipitaceous grass endophytes (Class I - endophytes) (known as C-endophytes). They are characterised by a close association with their host plants because of their vertical transmission via seed infections and production of specific alkaloids toxic to insects (Saikkonen *et al.*, 2002). According to Rodriguez *et al.* (2009), these endophytes belong to type I and Class I C – endophytes and may be classified as Class II C - endophytes when they colonise both above – and below – ground tissues of their respective host plants.

Their ability to colonize both above- and below-ground plant tissues may explain their proficiency at the production of toxins effective in degrading and killing insects, particularly in free-living *Cordyceps* taxa (Rodriguez *et al.*, 2009). However, EPF-type endophytes residing within the Cordycipitaceae family and Hypocreales order (Sung *et al.*, 2007), classified as Class II endophytes within Ascomycota fungi division (Inglis *et al.*, 2001; Rehner and Buckley, 2005) share major precursors used by widespread grass endophytes (Rodriguez *et al.*, 2009). However, *Beauveria bassiana* (Ascomycota: Hypocreales) could be in association with the Poaceae, but knowledge about its association with cereal crops including wheat is limited. Such lack of knowledge became a driver for initiation of the current study to investigate the impact of the endophytic association of *B. bassiana* with selected SA wheat cultivars.

2.4 Inoculation of host plants

Inoculation of host plants with endophytic fungi is an acceptable integrated pest management (IPM) strategy that mitigates systemic plant resistance against enemy attacks. Fungal entomopathogens are explored for inoculation of plants because they potentially colonise plants endophytically, and considerably regulate insect population as mycopesticides (Hajek, 1997; Vega *et al.*, 2012). In several studies, *B. bassiana* were reported as endophytes that colonise different agronomic crops (Table 2.3), following successful inoculation.

Ideally, upon host plant inoculation, endophytic inoculum grows and penetrates through interspaces between parenchyma cells or in the xylem vessel leading to systemic growth of the hyphae and subsequent colonisation of the entire plant (Vidal and Jaber, 2015). The fungal entomopathogen *B. bassiana* colonises plants either naturally or after various inoculation techniques (Vega, 2008; McKinnon *et al.*, 2017).

2.4.1 Inoculation techniques

Different inoculation techniques have been explored in different studies to investigate the ease for endophytic entomopathogenic plant colonisation. The choice of inoculation method should ideally match the niche of the target herbivore or plant pathogen and should also target the intended location of the endophyte in a plant (below or under-ground plant tissues) (Parsa *et al.*, 2013). As highlighted by Posada *et al.* (2007), effective colonisation varies between crops depending on the technique used. Foliar spray of coffee favoured leaf colonisation whereas soil drenches support root colonisation. In addition, Parsa *et al.* (2013) also reported different inoculation methods from which *B. bassiana* was tested for endophytic establishment in various host plants. These methods include soil drenching, seed immersion and coating, root and rhizome immersion, stem injection, foliar spray and flower spray. However, leaf spray and soil drenching methods effectively facilitated plant colonisation in their trial crop, beans in their study. In the review presented by McKinnon *et al.* (2007), most of the techniques exploited, successfully established the *B. bassiana* endophytes in inoculated plants (Table 2.4).

Table 2.4: Information about three inoculation techniques used for establishment of *B. bassiana* in different studies (McKinnon *et al.*, 2017).

Inoculation techniques	Host plant species	Plant parts sampled	Surface sterilisation done	Reference
Seed treatment	Bean (<i>Vicia faba</i>)	Roots	Yes	Akello and Sikora (2012)
	Bean (<i>V. faba</i> , <i>Phaseolus vulgaris</i>)	Roots, leaves stems	Yes	Akutse <i>et al.</i> (2013)
	Bean (<i>V. faba</i>)	Roots, leaves stems	Yes	Akutse <i>et al.</i> (2014)
	Jute (<i>Chorchorus olitorius</i>)	Roots, leaves, stem, capsules	Yes	Biswas <i>et al.</i> (2012)
	White Jute (<i>Chorchorus capsularis</i>)	Roots, needles	Yes	Biswas <i>et al.</i> (2013)
	Pine (<i>Pinus radiata</i>)	Leaves	Yes	Brownbridge <i>et al.</i> (2012)
	Cotton (<i>Gossypium</i> spp.)	Seed	Yes	Castillo-Lopez and Sword (2014)
	Cotton (<i>Gossypium</i> spp.)	N/A	No	Castillo-Lopez and Sword (2015)
	Tomato (<i>Solanum</i> spp.)	Seedlings	Yes	Ownley <i>et al.</i> (2008)
	Cotton (<i>Gossypium</i> spp.)	Leaves, stem, roots	Yes	Powell <i>et al.</i> (2009)
	Opium poppy (<i>Papaver somniferum</i>)	Leaves, seeds	Yes	Quesada-Moraga <i>et al.</i> (2014)
Leaf spraying (Topical)	Sorghum (<i>Sorghum bicolor</i>)	Leaves, stems, roots	Yes	Tefera and Vidal (2009)
	Cocoa (<i>Conopomorpha cramerella</i>)	Pods	Yes	Amin <i>et al.</i> (2014)
	Corn (<i>Zea mays</i>)	Whorl parts, pith	No	Bing and Lewis (1991- 1993), Lewis <i>et al.</i> (2002)
	Date palm (<i>Phoenix dactylifera</i>)	Petioles	Yes	Gomez-Vidal <i>et al.</i> (2006)
	Artichoke (<i>Cynara scolymus</i>)	Leaves	Yes	Guesmi-Jouini <i>et al.</i> (2014)
	Cotton (<i>Gossypium</i> spp.) wheat, (<i>T. aestivum</i>), bean (<i>P. vulgaris</i>), corn (<i>Z. mays</i>), pumpkin (<i>Cucurbita maxima</i>)	Leaves	Yes	Gurulingappa <i>et al.</i> (2010)
	Grapevine (<i>Plasmopara viticola</i>)	Leaves	Yes	Jaber (2015)
	Squash (<i>C. pepo</i>)	Leaves	Yes	Jaber and Salem (2014)

Table 2.4 continued

Leaf spraying (Topical) continued	Rice (<i>Oryza</i> spp.)	Leaves, stems, roots, seeds	Yes	Jia <i>et al.</i> (2013)
	Opium (<i>P. somniferum</i>)	Leaves	Yes	Landa <i>et al.</i> (2013)
	Sorghum (<i>S. bicolor</i>)	Leaves, stems	Yes	Mantzoukas <i>et al.</i> (2015)
	Bean (<i>P. vulgaris</i>)	Leaves, stems	Yes	Parsa <i>et al.</i> (2013)
	Coffee (<i>Coffea arabica</i>)	Leaves, stems	Yes	Posada <i>et al.</i> (2007)
	Cocoa (<i>Theobroma cacao</i>), Coffee (<i>C. arabica</i>)	Leaves, stem, roots	Yes	Posada and Vega (2005, 2006);
	Coffee (<i>Coffea arabica</i>)	Leaves, stems, roots, pods	Yes	Posada <i>et al.</i> (2010)
	Opium poppy (<i>P. somniferum</i>)	Leaves, cotyledons	Yes	Quesada-Moraga <i>et al.</i> (2006, 2009)
	Tobacco (<i>Nicotina tabacum</i>)	Stems	Yes	Reddy <i>et al.</i> (2009)
	Corn (<i>Z. mays</i>), wheat (<i>T. aestivum</i>), Soybeans (<i>Glycine max</i>)	Leaves	Yes	Russo <i>et al.</i> (2015)
	Oil seed rape (<i>Brassica napus</i>), bean (<i>V. faba</i>)	Leaves	Yes	Vidal and Jaber (2015)
	Corn (<i>Z. mays</i>)	Leaves	Yes	Wagner and Lewis (2000)
Soil drenching	Cassava (<i>Manihot esculenta</i>)	Leaves, stems, roots	Yes	Greenfield <i>et al.</i> (2016)
	Bean (<i>P. vulgaris</i>)	Leaves, stems, roots	Yes	Parsa <i>et al.</i> (2013)
	Coffee (<i>C. arabica</i>)	Leaves, stems, roots	Yes	Posada <i>et al.</i> (2007)
	Sorghum (<i>S. bicolor</i>)	Leaves, stems, roots	Yes	Tefera and Vidal (2009)

Interestingly, of many techniques exploited, topical leaf sprays followed by seed treatment were the most commonly and widely used in most studies, with few reports about soil drenching (Greenfield *et al.*, 2016). However, few studies employed all three techniques simultaneously (Tefera and Vidal, 2009) including the current study. Generally, successful colonisation of plants could be influenced by the nature of the cultivar and growth media. According to Vidal and Jaber (2015), specific cultivar colonisation is enhanced when soil conditions are free from endophyte inhibitors.

2.4.2 Impact of induced systemic resistance by EPF

Microbial soil communities also known as microbiome are perceived for their potential to control plant health as they employ an array of mechanisms that function prominently to suppress diseases that are prevalent in soils (Bakker *et al.*, 2013). These suppression mechanisms include competition for nutrients, antibiosis, induced systemic resistance (ISR) and induced tolerance to abiotic stresses (Yang *et al.*, 2008). Of all these mechanisms, ISR was explored as biological control strategy and was found to be the best mechanism that explains disease suppression in most soils (Mazzola, 2002). The term induced systemic resistance is generic for the induced state of resistance in plants triggered by biological or chemical inducers, which protects non-exposed plant parts against future attack by pathogenic microbes and herbivorous insects (Kuc, 1982). This resistance is characterized by activation of latent defense mechanisms that are expressed upon a subsequent challenge from a pathogen or insect herbivore. Induced resistance is not expressed only locally at the site of induction but also systemically in

plant parts that are spatially separated from the inducer, hence the term ISR. Direct activation of defense mechanisms includes increased basal levels of defense-related compounds, or the priming of the plant defensive capacity (Martínez-Hidalgo *et al.*, 2015). Priming is an activity that enhances plant capacity to rapidly and effectively improve defence responses towards biotic and abiotic stresses (Conrath *et al.*, 2006). Effective priming demonstrates accelerated defence-related gene expression to enhance high level of resistance against pathogen attack, insect herbivory or broad spectrum of attackers (Conrath *et al.*, 2006). In the latter, the presence of the challenge triggers some major changes upon induction, with more efficient activation of defense mechanisms elicited upon attack. Thus, priming is a cost-effective way of increasing plant resistance (Conrath *et al.*, 2006; Pastor *et al.*, 2013).

Dose response studies for biological control agents also confirmed a need for high microbial populations to ensure effective control of diseases (Johnson and DiLeone, 1999). According to Van Pelt *et al.* (2008), to effectively elicit ISR, high population densities of a specific biocontrol strain are introduced into the soil or rhizosphere, usually around 10^7 colony forming units (cfu) per gram of root. Bakker *et al.* (2013) also reviewed and confirmed the ability of specific rhizosphere (root associated) microorganisms to elicit induced systemic resistance against pathogens.

2.5 Detection methodology

Detection of endophytes from inoculated plants is a critical endeavor to ascertain colonisation success. Colonisation following inoculation of plants is confirmed through several techniques such as microscopy, direct isolation of fungus from plant tissues using culture media (culture dependent) and molecular detection of fungal DNA from plant tissues using PCR (culture independent) (McKinnon *et al.*, 2017).

2.5.1 Microscopy

Endophytic detection of *B. bassiana* using light and electron microscopy has been employed in the previous studies to determine conidia germination on the leaf surface (Wagner and Lewis, 2000; Quesada-Moraga *et al.*, 2006). Visualisation and identification of endophytes using morphological traits such as spore shape and size (Glare and Inwood, 1998), may be complicated with this method. Scanning electron microscopy has been deployed to observe endophytic plant colonisation (Sasan and Bidochka, 2012). However, lacking sensitivity and specificity in microscopy makes it a hurdle to identify endophytes within the plant tissues, hence such studies are rare (McKinnon *et al.*, 2017).

2.5.2 Physical recovery

Traditional culture identification methods relied on selective growth plating followed by morphological characterisation, sugar fermentation and haemolytic properties of

microbial species. Surface sterilisation of sampled plant parts usually precedes the plating technique to ensure adequate removal of epiphytes from the surface. Different sterilization times, concentrations and volumes could be used according to the thickness and the texture of wheat leaf surface (Larran *et al.*, 2002a). Media plating of the third rinse water to ensure accuracy of surface sterilisation method is another method for confirmation of endophytic colonisation. However, these methods are lengthy and lack potential sensitivity and rapidity to allow identification within short periods (Gasarov *et al.*, 2005). Thus, more rapid tests were developed to detect and confirm endophytic activities of *B. bassiana* based on molecular techniques.

2.5.3 Molecular techniques

Identification of *B. bassiana* is however, difficult using morphological traits such as spore shape and size (Glare and Inwood, 1998). As a result, this has warranted the development of molecular typing tools for identification of *B. bassiana* isolates (Hegedes and Khachatourians, 1996; Enkerli and Widmer, 2010; Rehner *et al.*, 2011). Most dedicated techniques to studying genetic diversity of EPFs were PCR, protein assays and DNA/RNA probes (such as restriction fragment length polymorphism) (Gaitán-Solís *et al.* 2002), and internal transcribed spacers of the ribosomal DNA (Ghikas *et al.* 2010). Previously, microsatellites were employed to analyse genetic diversity among *B. bassiana* isolates (Castrillo *et al.* 2003; Meyling and Eilenberg, 2006).

In recent studies, positive colonisation of plants by endophytes was confirmed through re-isolation from potato dextrose agar (PDA) media plating and PCR analysis (Castillo-Lopez *et al.*, 2014). In conjunction with this, PCR detection of a *B. bassiana* strain through the two-step nested specific-PCR protocol using species specific primers and subsequent sequencing of ribosomal ITS region of *B. bassiana* strain EABb 04/01-Tip also confirmed colonisation of opium plants (Quesada-Moraga *et al.*, 2014). Molecular detection of *B. bassiana* strains using the specific-PCR protocol and sequencing of the ITS region of *B. bassiana* strain were also used to confirm colonisation of opium plants (Quesada-Moraga *et al.*, 2014).

However, choice of these techniques relies on their specificity, sensitivity, and rapidity towards tested genotypes. Based on our understanding, newly developed techniques based on quantitative PCR (qPCR) and loop-mediated isothermal amplification (LAMP) assays are highly sensitive, specific, and rapid (Thekisoe *et al.* 2005, 2007; Lau *et al.* 2010; Karthik *et al.* 2016), but rarely explored for identification of fungal environmental samples and endophytes.

2.6 General safety of *B. bassiana* as a biological control agent

With an increasing interest in biological control of insect pests, the major hurdle was a limited knowledge on safety of insect pathogens on other organisms. This concern suggested a necessity for implementing the safety or risk assessment of insect

pathogens (Heimpel, 1971). Subsequent designation of registration guidelines prior to commercialisation of fungal BCAs particularly those that secrete fungal secondary metabolites was considered (Strasser *et al.*, 2000). In the wide range of fungal metabolites secreted, some play important roles in medicine and are incorporated in research tools (Vey *et al.*, 2001). Interestingly, initial investigations based on toxicity of BCAs prior to formulation and registration of commercial products were undertaken against several mammals (including rats) and no toxic or pathogenic reactions were noticed (Schaerffenberg, 1968). Further experiments to evaluate infectivity or toxicity of *B. bassiana* commercial strains GHA and ATCC 74040 on rats were conducted and no toxicity was documented (Goettel and Jaronski, 1997). In agreement with the above-mentioned results, Copping (2004) observed no infectivity or pathogenicity of *B. bassiana* strain ATCC 74040 in rats 21 days post treatment. While recent investigations suggested that the absence of viable *B. bassiana* conidia in tested birds could be due to the high body temperature (40°C). The phenomenon was believed to have prevented the conidia to germinate and cause infection or release any kind of toxins in the birds' body (Haas-Costa *et al.*, 2010). Safety of *B. bassiana* on non-target organisms was recently proven on earthworms and other phoretic hosts (Shapiro-Ilan and Brown, 2013). In the latter, commensalism or phoresis between *B. bassiana* and earthworms assisted in dispersal of this EPF populations in various ecosystems, thereby assisting in regulation of various pests. This further asserts that *B. bassiana* is an environmentally friendly biocontrol agent.

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CHAPTER 3: PATHOGENICITY OF *BEAUVERIA BASSIANA* (HYPOCREALES: CORDYCEPTACEAE) AGAINST THE RUSSIAN WHEAT APHID, *DIURAPHIS NOXIA* (HEMIPTERA: APHIDIDAE)

3.1 Introduction

In SA, bread wheat, *Triticum aestivum* L. (Poaceae), serves as a host plant for several aphid pests, including the notorious Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov) Hemiptera: Aphididae (Prinsloo and Tolmay, 2017). As with other *r*-selected organisms (MacArthur and Wilson, 1967), aphids are characterised by a high reproduction rate and short generation period, leading to exponential population increases (Hales *et al.* 1997); often exceeding economic threshold levels. Since its first occurrence in SA in 1978 (Walters *et al.*, 1980), the RWA has almost exclusively been controlled with insecticides, until the release of the first resistant wheat cultivar (Tugela-DN) in 1992 (Marasas *et al.*, 1997). Although the use of host plant resistance is now a well-adopted strategy in SA, the current existence of at least four resistance-breaking RWA biotypes (reported dates: 1978 [RWASA1], 2005 [RWASA2], 2009 [RWASA3] and 2011 [RWASA4]) raises serious concerns, especially amidst climate fluctuations (Jankielsohn, 2017). Should this resistance collapse, wheat producers may again resort to the use of insecticides, which carry an environmental as well as human and animal health risk. Moreover, under an insecticide selection-pressure scenario, survival of viviparous parthenogenetic female aphids implies the production of offspring with the same genetic constitution as the genitor (Ogawa and Miura, 2014), hence contributing

to the risk of resistant populations developing over time. The emergence of aphid biotypes in different plant-aphid crop systems is devastating (Smith and Chuang, 2014).

Therefore, to fully capitalise on host plant resistance, an integrated pest management (IPM) approach should ideally be pursued, whereby the natural enemy complex as a biological control component is also promoted. Diverse strains of EPFs such as *Lecanicillium* sp. (Jung *et al.*, 2006; Fadayivata *et al.*, 2014), *B. bassiana* (Hatting *et al.*, 2004; Quesada-Moraga *et al.*, 2006), *M. anisopliae* (Wright *et al.*, 2004), *Isaria* sp. (Shia and Feng, 2004), and *Nomuraea rileyi* (Devi *et al.*, 2003) have been used for the management of aphids and many other pests. In turn, various fungal isolates demonstrated successful control of different aphid species (Alavo *et al.*, 2002; Hatting *et al.*, 2004; Kim *et al.*, 2007; Hesketh *et al.*, 2008).

The natural enemy complex associated with the RWA in SA comprises of indigenous and exotic predators, parasitoids and entomopathogens (Hatting, 2002). Locally, the notion of integrating the EPF, *Beauveria bassiana* (Hypocreales: Cordycipitaceae) with host plant resistance, was explored by Hatting *et al.* (2004), recording *ca.* 65% fewer aphids in fungus-treated plots compared to controls. Although in the former study a commercial strain (*B. bassiana* strain GHA) was used, local indigenous fungal strains are not yet fully exploited for RWA control. Based on Milner (1992) isolates obtained from the fields on target pests are often the best candidates for mycoinsecticide development because their behaviour is already stable under field conditions. However,

performance of fungal strains not directly associated with natural field-mycoses in the target pest, may be screened through biological assays (bioassays) to measure their physiological host range (Finney 1979; Hatting and Wraight, 2007). The measurement of fungal strain performance or virulence, is generally quantified in terms of the median lethal concentration (LC_{50}) and/or median lethal time (LT_{50}) required to kill 50% of the target insect population and can be impacted upon by various biotic and abiotic factors (Hatting and Wraight, 2007).

In this study, a candidate *B. bassiana* strain (PPRI 7598) was selected following screening of 21 indigenous *B. bassiana* isolates for insect virulence. Selection was based on >95% mortality of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) and >90% germination. In SA, no further reports have yet been documented on the efficacy of indigenous *B. bassiana* isolates against RWA after the evaluation of PPRI 7176 by Hatting (2002). Objectives of this study were (1) to compare pathogenicity of the two indigenous *B. bassiana* strains (non-commercial, PPRI 7598 versus commercial, PPRI 7861) against RWA and select the most virulent isolate, and (2) to compare virulence of the selected isolate towards RWA following passage and re-isolation from an insect ('IN') versus plant ('PL') host background.

3.2 Materials and Methods

3.2.1 Fungal isolates

Fungal isolates used in this study were obtained from the Agricultural Research Council – Small Grain Fungal Culture Collection in Bethlehem, SA. Pre-screening of strains involved 21 *Beauveria bassiana* isolates, all recovered from soil or different insect taxa. Isolates were first cultured on Sabouraud dextrose agar amended with 1% yeast extract (SDAY Biolab, Merck, Longmeadow, Modderfontein, 1645) in 65 mm petri dishes incubated at 22 ± 3 °C in complete darkness for 7-14 days. Dry conidia were harvested from 14-day-old cultures (21 isolates) using a sterile camel-hair brush. Conidial viability was assessed based on >95% germination after incubation for 24 hours at 22 ± 3 °C in total darkness. Random counts were performed by microscopically inspecting 100 conidia from three different areas per plate at 40x magnification using light microscope (Nikon Optiphot, Japan.). Secondly, isolates were also pre-screened for pathogenicity against final instar *Galleria mellonella* larvae through injection (Inglis *et al.*, 2012) with a 0.01 ml fungal suspension (*i.e.*, 0.03 g pure conidia suspended in 10 ml sterile water, amended with 0.01% Break-Thru® [polyether-polymethylsiloxane-copolymer surfactant; Goldschmidt Chemical Corporation, Hopewell, VA, USA]) per isolate (five larvae injected per isolate; totalling 105 larvae; data not shown). One isolate showing highest mortality (*i.e.*, PPRI 7598 with 100% mortality; data not shown) was selected for further comparison with *B. bassiana* strain PPRI 7861, the active ingredient in the commercial myco-insecticide, Eco-Bb® (Agri-Intel, 2017).

3.2.2 Aphid rearing

The RWASA1 colonies were reared in the ARC-Small Grain insectary unit. An aphid colony was established by infesting insect-free wheat seedlings (Tugela) at four-leaf growth stage (Hatting and Wraight, 2007). Aphids were reared in gauze cages in a glasshouse at $22 \pm 3^{\circ}\text{C}$, $40 \pm 4\%$ relative humidity (RH) and natural light. Aphids were monitored until they reached the adult stage prior to use in bioassays.

3.2.3 Bioassay protocols

3.2.3.1 Preparation of conidial suspension

Beauveria bassiana isolates were grown on SDAY and incubated at $22 \pm 3^{\circ}\text{C}$ for 14 days. Conidia were harvested with a sterile camel-hair paint brush and immersed in sterile distilled water containing 0.01% Break-Thru®. Conidial suspension was homogenised with a vortex for 5 minutes and concentrations adjusted as described under 3.2.3.2 below.

3.2.3.2 Comparison of two indigenous B. bassiana strains (non-commercial versus commercial) against RWA

The two *B. bassiana* strains, PPRI 7598 and PPRI 7861, were compared for virulence against RWA according to the assay protocol of Hatting and Wraight (2007). Conidial suspensions were adjusted to 1×10^8 (designated "High") and 5×10^7 ("Low") conidia ml⁻¹.

¹, using an improved Neubauer haemocytometer (Marienfeld, Germany). In addition, the contact insecticide Aphox[®] WG (Pirimicarb), was included as chemical standard at a concentration of 0.5 g/L. Age-related adult apterae aphids (biotype RWASA1) were prepared as described under 3.2.2. with 5 replicate groups of 20 aphids each (100 aphids in total) allocated to each treatment (x5) and control (total 600 aphids). Control aphids were sprayed with sterile water containing 0.01% Break-Thru[®] surfactant. All treatments and the control were sprayed with 5 ml aliquots inside a Burgerjon precision spray tower (Burgerjon, 1956). During spraying, a Petri dish containing 1.5% water agar was placed adjacent to the aphids on the same radial dimension to quantify the actual number of conidia deposited per mm². As per protocol by Hatting and Wraight (2007), inoculated aphids were maintained on wheat seedlings (cultivar Tugela; 4-leaf stage) under glasshouse conditions at 25 ±2°C, 40 ±5% RH and natural light for 7 days post-inoculation. Mortality was assessed daily for the duration of the assay and all dead aphids were placed on 1.5% water agar and incubated at 22 ±3 °C to facilitate the development of overt mycosis. Three independent series of assays were conducted, at least seven days apart. Data were corrected for control mortality according to Schneider-Orelli's formula (Schneider-Orelli, 1947).

$$\text{Corrected \%} = \left(\frac{\text{Mortality \% in treated} - \text{Mortality \% in control}}{100 - \text{Mortality \% in control}} \right) * 100$$

In addition, the mean time of mortality was also calculated by dividing the number of aphids, dying on a given day after treatment, by the total mortality after 7 days. This

value was then multiplied by the respective day, and values for days 1 – 7 were summed to produce a weighted average time of mortality for each treatment. Log transformation was applied to the average mortality for each day. The experimental design was completely randomised with five treatments (2 concentrations per each of the two isolates + chemical standard) and control. Each treatment included five replicates of 20 aphids each (100 aphids) and the entire assay was repeated thrice (1200 aphids in total).

*3.2.3.3 Virulence of *B. bassiana* to RWA following passage and re-isolation from an insect ('IN') versus plant ('PL') host background*

The most pathogenic strain (PPRI 7598), selected under section 3.2 above, was passed through and re-isolated from the target insect (*D. noxia*; via topical inoculation as described above) and wheat (*T. aestivum* cultivar Tugela; via seed imbibition with a conidial suspension containing 1×10^8 conidia ml⁻¹; cf. protocol by Akello and Sikora (2012). For fungal re-isolation from plants, sections of leaf, root and stem material were excised from 3-week old seedlings, surface sterilised and plated on SDAY as per protocol of Akello and Sikora (2012). Bioassays were performed, as above, with four treatments, *B. bassiana* "IN" and "PL" (at 1×10^8 conidia ml⁻¹), Aphox® and control (*i.e.*, 5 replicate groups of 20 aphids per group x 4 = 400 aphids). Mortality was assessed daily for the duration of the assay and all dead aphids collected and placed on 1.5% water agar and incubated at $22 \pm 3^\circ\text{C}$ in total darkness to facilitate the

development of overt mycosis. Three independent series of assays were conducted seven days apart.

3.2.4 Statistical analysis

Data were corrected for control mortality according to Schneider-Orelli's formula (Schneider-Orelli, 1947) and therefore the cumulative mortality was calculated. For the mean time of mortality, Log transformation was applied to the average mortality for each day prior to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). Combined analysis of trials (assays) was performed after trial variances had been verified as homogeneous by the Levene test (Levene, 1960). Repeated measurements of the data over days were treated, combined and added as a subplot factor in a split plot analysis of variance (Little and Hills, 1978). Shapiro-Wilk test was performed on the standardised residuals from the model to verify normality (Shapiro and Wilk, 1965). Fisher's protected least significant difference (LSD) was calculated at the 5% level to compare treatment means (Ott and Longnecker, 2001). A probability level of 5% was considered significant for all significance tests.

3.3 Results

3.3.1 Comparison of two indigenous *B. bassiana* strains (non-commercial versus commercial) against RWA.

While the chemical standard induced 100% mortality within 24h, both *B. bassiana* strains PPRI 7598 (non-commercial) and PPRI 7861 (commercial strain) were proved pathogenic to *D. noxia*. Both strains caused the highest cumulative Schneider Orelli-corrected mortality at the concentration of 1×10^8 conidia ml^{-1} (mean deposition of 2041 ± 65 and 1962 ± 53.46 conidia mm^{-2} , respectively: Table 3.1).

Table 3.1: Concentrations of deposited *Beauveria bassiana* conidia sprayed onto aphids inside Burgerjon Precision spray tower during three trials.

Fungal isolate	Concentration (Conidia ml^{-1})	Observed deposition (Conidia mm^{-2})	Observed deposition mean (Conidia $\text{mm}^{-2} \pm \text{SE}$) (CV)	Meantime of mortality (days)
PPRI 7598	1×10^8	1963, 1989, 2171	2041 ± 65.43 (6%)	4.51 ± 0.06
	5×10^7	874, 956, 855	895 ± 30.98 (6%)	4.34 ± 0.06
PPRI 7861	1×10^8	1892, 2067, 9127	1962 ± 53.46 (5%)	4.26 ± 0.07
	5×10^7	893, 910, 853	885 ± 16.89 (3%)	4.23 ± 0.06

The superior performance by PPRI 7598 induced the highest corrected mortality of 55.64% at 1×10^8 conidia ml^{-1} (observed 7 DPI (Fig. 3.1a) significantly higher ($P < 0.05$) than the 43.1% recorded with PPRI 7861 (commercial strain) at the same conidial concentration (Fig.3.1a). The interaction between time (days) and treatment was significant ($F = 242.01$; $df = 5$; $P \leq 0.0001$), with an increase in aphid mortality over time (Fig. 3.1a). During the first 4-day incubation period, aphid survival remained constant but showed a notable increase in mortality over time. At the high concentration, PPRI

7861 showed a significantly shorter ($P < 0.05$) mean time of mortality (4.26 ± 0.07 days) compared to PPRI 7598 (4.51 ± 0.06 days), but no significant difference was seen between the two lower concentrations.

The level of overt mycosis developing on RWA cadavers with PPRI 7598 (61.6%; $n = 63$) was significantly higher ($P < 0.05$) as compared to that with PPRI 7861 (52.1%; $n = 53$) at 1×10^8 conidia ml^{-1} . No differences were noted at the lower concentration (Fig. 3.1b).

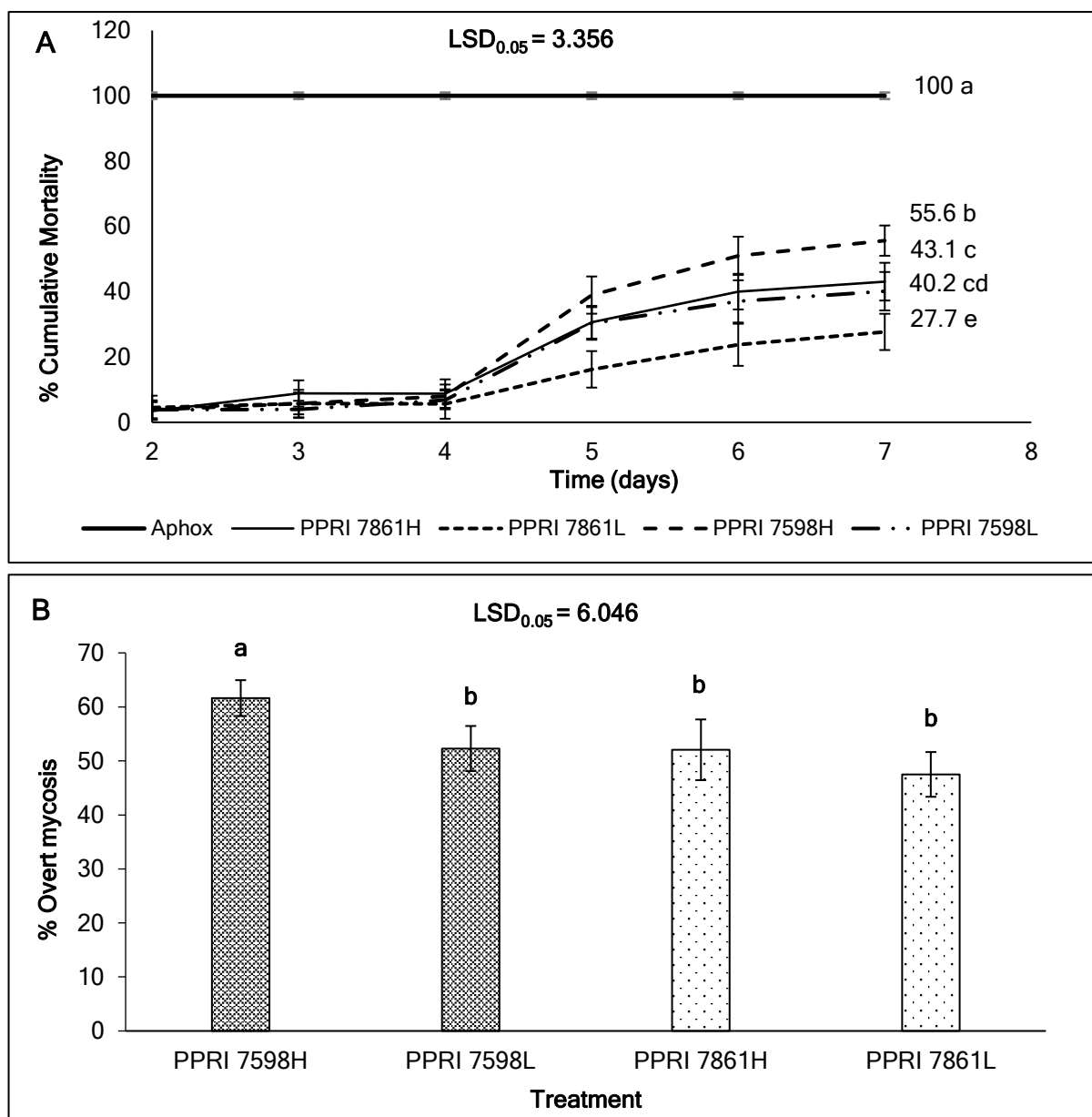


Figure 3.1: Comparative virulence of PPRI 7598 and PPRI 7861 to Russian wheat aphid. **(A)** Cumulative mean percentage Schneider-Orelli - corrected mortality of RWA over 7 days. Day-7 mortalities followed by the same letter are not significantly different at the 5% test level; **(B)** Percentage overt mycosis 7 DPI. Columns with the same lowercase letters are not significantly different ($P < 0.05$). The bars (Means $\pm$ SEM) marked with different letters indicate significant differences at P value < 0.05 . H indicates high concentration and L indicates low concentration.

3.3.2 Virulence of *B. bassiana* against RWA following inoculation with an insect-derived ('IN') versus plant-derived ('PL') isolates

The conidial depositions (number of deposited propagules per square millimeter) for PPRI 7598IN and PPRI 7598PL, were 1820 ± 68 conidia mm^{-2} and 1958 ± 60 conidia mm^{-2} , respectively. Slightly higher mortality was caused by the insect (56.9%) versus plant (50.1%) backgrounds, albeit not significantly different at the 5% test level with LSD of 7.562 (Fig. 3.2a). Moreover, a significantly shorter mean time of mortality was recorded for the insect-derived (4.14 ± 0.04 days) compared to plant-derived (4.58 ± 0.08 days) isolates ($\text{LSD}_{0.05} = 0.247$), while the level of overt mycosis recorded with PPRI 7598IN (58.2%; $n = 60$) was higher ($\text{LSD}_{0.05} = 6.488$) compared with PPRI 7598PL (47.9%; $n = 55$) (Fig. 3.2b)

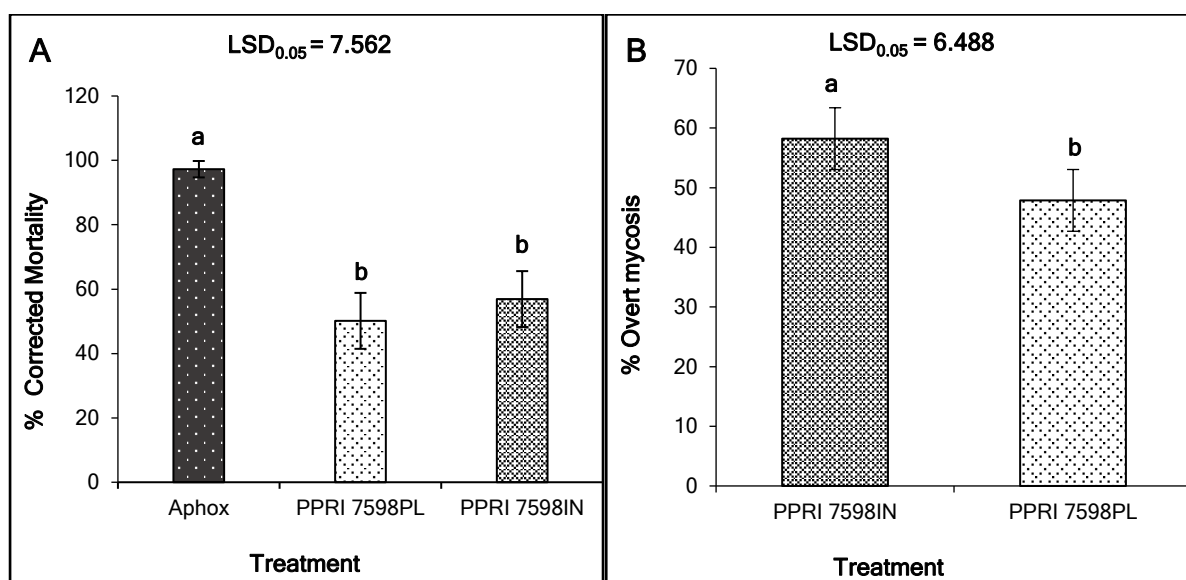


Figure 3.2: RWA mortality parameters presented as: **(A)** Mean percentage Schneider-Orelli - corrected mortality of RWA, 7 DPI, caused by the chemical standard and two background isolates of PPRI 7598 **(B)** mean percentage overt mycosis by the two PPRI 7598 backgrounds 7 DPI. The bars (Means±SEM) marked with different letters indicate significant differences at P value <0.05.

3.4 Discussion

After inoculation, insect mycosis follows a typical 3-phase pattern of disease progression, characterised by (1) the incubation period, (2) first sign and/or symptom development (*i.e.*, pre-mortem period) and (3) actual mortality (Tanada and Kaya, 1993). Generally, LC_{50} and LT_{50} parameters are calculated through probit analyses for quantification of virulence (Finney, 1971). However, where mortalities did not span the 10-90% range (ideal) or where comparative mortalities are investigated by means of a single-dose assay, the mean time of mortality can serve as an additional virulence indicator (Poprawski *et al.*, 1999). Although the commercial strain PPRI 7861 showed a shorter mean time of mortality (superior virulence), selection of PPRI 7598 was ultimately based on the higher 7-day cumulative mortality of 55.6%, coupled with the 10% higher level of overt mycosis developing on cadavers. Nevertheless, the mean time of mortality at $2\,041 \pm 65$ conidia mm^{-1} , was longer than the 2.3 ± 0.6 days reported with the highly virulent *B. bassiana* strain GHA at a comparable $2\,139 \pm 424$ conidia mm^{-1} , employing the same assay methodology (Hatting and Wraight, 2007). Our average mean time of mortality for the two isolates at the higher conidial concentration was 4.3 ± 0.15 days, comparable with the average 4.1 ± 0.4 days reported for six isolates of *B. bassiana* against the brown citrus aphid, *Toxoptera citricida* (Kirkaldy) (Homoptera: Aphididae) (Poprawski *et al.*, 1999). Interestingly, a shorter average period of 3.5 ± 0.4 days was recorded with six isolates of *Paecilomyces fumosoroseus* (Wize) Brown and Smith (now *Isaria fumosorosea*; Sung *et al.*, 2007) against *T. citricida* in the latter study. However, regardless of the longest mean time of mortality encountered with our

strains, the two were pathogenic to the target insect (RWA) with considerable variation in their virulence. Although not included in this study, *I. fumosorosea* also outperformed *B. bassiana* against RWA (Vandenberg, 1996), perhaps underscoring the need for further research on this EPF for aphid biocontrol; also from an endophytic perspective (Vega, 2008). Varying susceptibility of RWA towards *B. bassiana* strains encountered in our study confirms the observations that, the same insect host can resist certain strains of *B. bassiana* but be highly sensitive to others (Todorova *et al.*, 1994; 1996). A Similar scenario with insect mortality becomes evident when certain strain induces no mortality to one insect host but showing higher pathogenicity to another insect of the same order (Todorova *et al.*, 1994).

Virulence of an EPF can be affected by the insect host background (Hatting, 2005), continuous *in vitro* subculturing (Vandenberg and Cantone, 2004), constituents of the growth medium (Safavi *et al.*, 2007; Ortiz-Urquiza *et al.*, 2016) and perhaps more importantly from an endophytic perspective, the host plant on which the target insect feeds (Ramoska and Todd, 1985; Poprawski and Walker, 2000; Thungrabeab *et al.*, 2006). Artificial introduction of *B. bassiana* as endophyte into wheat or any other host plant implies subjecting the fungus to a different growing environment in terms of nutrients, pH or phytochemicals.

Generally, passage of an EPF through an insect host is seen as advantageous, *i.e.*, a means of either enhancing or restoring virulence (Song and Feng, 2011). However, the

effect on virulence following passaging through a plant has remained largely unexplored, with this study being the first to report on such a phenomenon. Use of plant-passaged conidia caused slightly lower (significant only at the 10% test level) aphid mortality, a significantly longer mean time of mortality as well as a lower level of overt mycosis, suggesting some level of impediment. Such level of impediment concurs with the notion by Vidal and Jaber (2015) that antagonistic interactions between already established endophytes (Crous *et al.*, 1995; Larran *et al.*, 2002, 2007; Vujanovic *et al.*, 2012; Comby *et al.*, 2016; Grudzinska-Sterno *et al.*, 2016) (note, axenic plants were not used in our study, although possible through embryo excision; see Robinson *et al.*, 2016) and artificially introduced EPFs are the rule rather than the exception. Moreover, plants may also upregulate their defence mechanism (Rojas *et al.* 2014) through release of phytochemicals. Notably, *T. aestivum* harbors a phytochemical complex (rutin, chlorogenic acid, tocopherol, chlorogenic acid, and gallic acid) (Rajoria *et al.*, 2015), potentially acting in an antagonistic fashion against pathogens. Likewise, such defenses may have affected *B. bassiana* passaged through the host plant, thus leading to slightly compromised virulence against RWA. On a positive note, the existence of such bioactive compounds in a host plant or as plant extracts could buffer against wheat phytopathogens of economic importance like *Fusarium graminearum* (Fusarium head Blight), *Tilletia indica* (Karnal Bunt) and *Puccinia triticina* (leaf rust). However, molecular techniques could better quantify levels of fungal colonisation following inoculation.

In SA, *B. bassiana* is used as an active ingredient in some commercial bioproducts available for crop pest control (Hatting *et al.*, 2018). These products, however, are all registered for topical application onto crops/insects. The endophytic ability of these different (commercial) strains remains unexplored and may thus hold potential for exploitation towards induced systemic resistance to insects and plant pathogens (Vega, 2018).

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CHAPTER 4: ENDOPHYTIC ESTABLISHMENT OF *BEAUVERIA BASSIANA* IN WHEAT THROUGH DIFFERENT INOCULATION TECHNIQUES AND ITS EFFECT ON PLANT GROWTH

4.1 Introduction

The ubiquitous nature of the EPF, *Beauveria bassiana* (Hypocreales: Cordycipitaceae) within global agro-ecosystems, renders it suitable for exploitation during Conservation Biological Control (CBC) (Fuxa, 1998), as proposed by Meyling and Eilenberg (2007). In that review, it was reported that *B. bassiana* associates with one of three basic substrates: soil, insects or plants. In the latter association, its exploitation as endophyte (Vega, 2008) for induced systemic resistance to biotic and/or abiotic stressors (Rodriguez *et al.*, 2009), is becoming an attractive possibility. Moreover, as host plant, *T. aestivum* appears endophytic-friendly, both as natural host (Sieber *et al.*, 1988; Crous *et al.*, 1995; Larran *et al.*, 2002, 2007; Vujanovic *et al.*, 2012; Comby *et al.*, 2016; Grudzinska-Sterno *et al.*, 2016) and when artificially inoculated (Dingle and McGee, 2003; Gurulingappa *et al.*, 2010; Russo *et al.*, 2015; Sánchez-Rodríguez *et al.*, 2015; Sánchez-Rodríguez *et al.*, 2018).

In recent studies, different inoculation methods artificially induced artificial establishment of endophytes in different crops (Posada *et al.*, 2007; Tefera and Vidal, 2009; Parsa *et al.*, 2013, Greenfield *et al.*, 2016; Jaber and Enkerli, 2016) including *T. aestivum*. As pointed out by Xiao *et al.* (2012), the endophytic ability of *B. bassiana* could

be supported by the lack of fungal endoxylanases (GH11) production; enzymes known to trigger plant immune responses and induction of necrosis of infected plant tissue (Noda *et al.*, 2010). Lack of GH11 in *B. bassiana* may therefore facilitate immune evasion, with subsequent endophytic establishment.

In a recent review on the use of EPF as endophytes in biological control, Vega (2018) found that 34 of the 85 papers covered (40%) examined plant responses to endophytism, covering 20 plant species including *T. aestivum*. Therefore, the notion of exploiting *B. bassiana* in a CBC approach (Meyling and Eilenberg, 2007) carries relevance, not only in terms of insect (and disease; see Busby *et al.*, 2016; Jaber and Ownley, 2018) suppression, but potentially also as plant growth-promoting factor (Behie and Bidochka, 2014; Sánchez-Rodríguez *et al.*, 2015; Liao *et al.*, 2017; Sánchez-Rodríguez *et al.*, 2018). In nature, the two primary 'sources' of *B. bassiana* inoculum is either insect- or plant-derived, while the historical frequency of recycling through either substrate, remains largely unknown. Host plant contact with such inoculum can be via seed/roots in the soil environment (Zimmermann, 2007) and/or via above ground phylloplanes (Meyling and Eilenberg, 2006; Howe *et al.*, 2016).

The current study was the first of its kind conducted to investigate the growth-response of South African wheat cultivars to endophytic *B. bassiana*, passaged through an insect versus plant substrate. Three inoculation techniques (seed imbibition, soil drenching and leaf spraying) were employed to simulate potential contact or endophytic mechanisms possible under field conditions.

4.2 Materials and methods

*4.2.1 Endophytic establishment through seed treatment with insect- versus plant-derived backgrounds of *Beauveria bassiana* in five South African wheat cultivars*

Beauveria bassiana strain PPRI 7598 used in this study was originally isolated from a soil sample collected in the Western Cape Province, SA. The strain was passaged through and re-isolated from the target insect, RWASA1 (designated "IN"), via topical inoculation (Inglis *et al.* (2012) and host plant wheat, *T. aestivum* (designated "PL") (cultivar Tugela; via seed imbibition). The two hosts were inoculated with a conidial suspension containing 1×10^8 conidia ml^{-1} (Akello and Sikora, 2012). For fungal extraction from plants, sections of leaf, root and stem material were excised from 3-week old seedlings, surface sterilised and plated on SDAY as per protocol of Bills (1996).

Seeds of three dryland-production wheat cultivars (Tugela, Elands and Gariep) and two irrigated-production cultivars (Kariega and Bavians), all developed by ARC-Small Grain, Bethlehem, SA, were used in this study. Seeds were surface sterilised according to the method of Akello and Sikora (2012) and each cultivar treated separately, with minor modifications, with the "IN" and "PL" backgrounds of *B. bassiana* 7598. Briefly, seeds were immersed in a 0.01% Break-Thru® (Polyether-polymethylsiloxane-copolymer surfactant; Goldschmidt Chemical Corporation, Hopewell, VA, USA) solution containing 1×10^8 conidia ml^{-1} and held on an orbital shaker at room temperature for 24 hrs to allow imbibition of the fungal suspension. Controls were handled in a similar

fashion, with 0.01% Break-Thru[®] solution without the fungal conidia. Inoculated and control seeds were planted in sterilised 295 ml plastic pots containing sterile soil ($\text{pH}_{[\text{KCl}]}$ = 4.8, P = 5.0 mg/kg, K = 125.0 mg/kg, Ca = 600 mg/kg, Mg = 203 mg/kg, Acid saturation = 4.2%) (heat-treated at $91 \pm 1^\circ\text{C}$ for 4 hrs). In each trial, two treatments ("IN" and "PL") comprised 1 125 plants (75 pots x 3 plants per pot x 5 cultivars) plus control (total 3 375 plants). Four independent trials were conducted, at least seven days apart. Plant colonisation by the two fungal backgrounds was evaluated at 7 and 14 days post inoculation (DPI). Segments from plant parts (roots, stems and leaves) were excised and surface sterilised according to the method of Bills (1996). Sterile parts were plated on *B. bassiana*-selective medium, containing 0.55 g/L dodine (guanidine) and 5 mg/L chlortetracycline antibiotic (Sigma – Aldrich, now Merck (Pty) Ltd, SA) as described by Chase *et al.* (1986). A total of 270 treated plant sections (9 segments from 3 plant parts x 5 cultivars x 2 observation times) were included in the trial. Plates with surface sterilised plant sections were incubated at $25 \pm 2^\circ\text{C}$ for 7-10 days and evaluated in comparison with the controls. The experimental design was completely randomised with five replicates. The treatment design was a split-split plot. The main plot treatments were the cultivars and first subplot was the fungal strains ("IN" and "PL"). The second subplot was plant parts (root, stem or leaf) while the third subplot was days post inoculation (7 and 14 DPI).

4.2.2 Effect of three inoculation techniques, i.e. seed imbibition, soil drenching and leaf spraying, on five plant growth parameters in five South African wheat cultivars

The five wheat cultivars were inoculated with *B. bassiana* 7598IN (selection based on higher level of endophytism compared to 7598PL), employing three different inoculation techniques, *viz.* seed imbibition, soil drenching and leaf spraying. Seed imbibition was performed as described above (section 4.2.1) on 1125 treated plants (75 pots x 3 plants per pot x 5 cultivars) and 1125 control plants (imbibition of water plus 0.01% Break-Thru®, only). For the soil drenching treatment, 5 ml of 1×10^8 conidia ml⁻¹ suspension was dispensed directly onto the soil around the stem-base of three 14-day old seedlings (*ca.* 1.7 ml per seedling), using a 5 ml stepper syringe (Socorex™ 411 Stepper). A total of 1125 plants (75 pots x 3 plants per pot x 5 cultivars) were included in the soil drenching treatment plus control (drenching of soil with water plus 0.01% Break-Thru®, only), totaling 2250 plants. For leaf inoculation, leaves of 1125 plants (75 pots x 3 plants per pot x 5 cultivars) were sprayed to the point of run-off, as per procedure of Rondot and Reineke (2018), with the same suspension concentration as above, using a hand-held atomizer. Control plants (1125) were sprayed with water plus 0.01% Break-Thru®, only. Prior to spraying, stem bases and the soil surface of treated pots were covered with aluminum foil to prevent inoculation of these areas (Posada *et al.*, 2007). Plants were maintained under glasshouse conditions at 25 ± 2 °C, 40 ± 5 RH and natural light for 42-45 days. All plants were evaluated for five plant growth parameters, *viz.*, shoot height (mm), root length (mm), fresh shoot biomass (g), fresh root biomass (g) and dry shoot biomass (g).

Three independent trials were conducted, at least seven days apart. The data included all factors (trial, cultivar, inoculation technique and treatment or fungal strains). The experimental design was completely randomized with five replicates and the treatment design was a split-plot with a two-factorial design as the main plot. The two factors were cultivars with five levels and inoculation techniques with three levels. The subplot factor was fungal strains with two levels (fungus treated and fungus free/control). The data were analysed according to the statistical procedures under section 4.2.3.

4.2.3 Statistical analyses

The homogeneity of trial variances was verified by Levene's test (Levene, 1960). The normality of the standardized residuals was confirmed using the Shapiro-Wilk test (Shapiro and Wilk, 1965). The data of the combined trials were subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Repeated measurements of the data over days were combined as a subplot factor in a split plot analysis of variance (Little and Hills, 1978). Fisher's protected least significant difference (LSD) was calculated at the 5% level to compare treatment means (Ott and Longnecker, 2001). A probability level of 5% was considered significant for all significance tests.

4.3 Results

4.3.2 Effect of three inoculation techniques on plant growth of five wheat cultivars

On average, treated plants showed a 71% increase in growth over controls, with individual growth parameter responses ranging from 29% to 104% (Table 4.1). Although seed imbibition ranked first (mean % improvement of 87.8%) among the three inoculation techniques employed (Table 4.1), leaf spraying resulted in a more consistent, high level of plant response, ranking first for four of the five parameters, *viz.*, fresh shoot biomass, dry shoot biomass, root length and shoot height.

Table 4.1: Effect of three inoculation techniques on seedling growth of five wheat cultivars (pooled) after 45 days.

Inoculation technique	Fresh shoot biomass (g)	Dry shoot biomass (g)	Fresh root biomass (g)	Root length (cm)	Shoot height (cm)	Grand mean
Seed imbibition	80.03b ¹ (2) ²	45.25b (3)	235.23a (1)	48.85b (2)	29.47ab (2)	87.77±42.22(1)
Soil drenching	76.96b (3)	75.35b (2)	39.48b (2)	32.20c (3)	25.84b (3)	49.97±12.20(3)
Leaf spraying	92.96a (1)	152.63a (1)	35.88b (3)	56.30a (1)	32.53a (1)	74.06±25.04(2)
LSD _{0.05}	9.10	33.95	23.32	6.74	5.99	
*G. mean	83.32±4.9	91.08±31.98	103.53±65.86	45.78±7.12	29.28±1.93	70.60±15.79

¹Means±SEM (% increase over controls) within columns followed by the same letter are not significantly different at the 5% test level

²Performance ranking within a given growth parameter (column) in parenthesis

*G. mean denotes the Grand Mean

However, when pairwise comparisons were performed among the cultivars for shoot height and fresh shoot biomass, the Fisher's unprotected LSD test (Hsu, 1996), did indicate differences. At the 5% test level, there were significant differences among the cultivars (Table 4.2) for three of the five parameters measured, *viz.*, dry shoot biomass ($F = 3.75$; $df = 4$; $P = 0.0072$), fresh root biomass ($F = 68.66$; $df = 4$; $P \leq 0.0001$) and root length ($F = 3.10$; $df = 4$; $P = 0.0192$), but non-significant for shoot height ($F = 2.31$; $df = 4$; $P = 0.0641$) and fresh shoot biomass ($F = 1.91$; $df = 4$; $P = 0.1154$).

This variation is further emphasized by the fact that for each growth parameter, a different cultivar ranked first (Table 4.2). Of particular interest was cultivar Kariega, showing a 260% increase in fresh root biomass (inoculation techniques pooled), and far exceeding the second-best responder (Gariép) at 81%. When considering the fresh root biomass-response of cultivar Kariega, as per inoculation technique, seed imbibition significantly outperformed leaf spraying and soil drenching with +672%, +54% and +7%, respectively ($LSD_{0.05} = 52.149$; $df = 90$) (Table 4.2).

Table 4.2: Cultivar growth response 45 days after inoculation (three techniques, pooled) with *B. bassiana* PPRI 7598.

Cultivar	Fresh shoot biomass (g)	Dry shoot biomass (g)	Fresh root biomass (g)	Root length (cm)	Shoot height (cm)	Grand mean
Tugela	86.84ab ¹ (2) ²	61.98b (5)	70.07bc (3)	50.39a (1)	26.63b (4)	59.18±10.07 (5)
Kariega	77.06b (5)	89.14b (3)	260.37a (1)	47.88a (3)	25.22b (5)	99.93±41.64 (1)
Baviaans	89.44a (1)	93.97b (2)	63.78bc (4)	37.59b (5)	28.87ab (3)	62.73±13.17 (4)
Elands	77.22b (4)	139.75a (1)	42.40c (5)	42.97ab (4)	29.62ab (2)	66.39±19.97 (2)
Gariep	86.03ab (3)	70.56b (4)	81.04b (2)	50.10a (2)	36.08a (1)	64.76±9.46 (3)
LSD_{0.05}	11.746 ³	43.832 ⁴	30.108 ⁴	8.705 ⁴	7.736 ³	-
*G. mean	83.32±2.58	91.08±13.51	103.53±39.71	45.79±2.44	29.28±1.89	70.6±6.91

¹ Means±SEM (% increase over controls) within columns followed by the same letter are not significantly different at the 5% test level

²Performance ranking within a given growth parameter (column) in parenthesis

³Fisher's unprotected LSD test

⁴Fisher's protected LSD test

*G. mean denotes the Grand Mean

4.3.1 Endophytic colonisation of wheat (inoculation of seeds through imbibition)

Beauveria bassiana was never successfully re-isolated from control plants in any experiment. The level of endophytic colonisation varied significantly among cultivars ($F = 24.81$, $df = 4$, $P \leq 0.0001$), between fungal backgrounds (7598IN vs 7598PL; $F = 17.48$; $df = 1$; $P \leq 0.0001$), among plant parts (root, stem and leaf; $F = 106.90$; $df = 2$; $P \leq 0.0001$) and between evaluation times (7 vs 14 DPI; $F = 168.15$; $df = 1$; $P \leq 0.0001$). The most colonised cultivar (plant parts pooled; $n = 96$ per cultivar; $LSD_{0.05} = 4.44$) was Baviaans (33.54%), followed by Tugela (31.34%), Kariega (27.87%), Gariep (25.67%) and Elands (21.28%). A significantly higher ($P < 0.05$) mean level of colonisation (plant parts combined) was recorded with 7598IN (29.74%; $n = 240$) as compared to 7598PL (26.13%; $n = 240$) (Fig. 4.1). The highest level of colonisation (44.15%) was recorded in roots with 7598IN at 7 DPI, statistically outperforming all other treatments (Fig. 1).

Generally, leaf colonisation was low, ranging from 12% to 28%. Recovery of *B. bassiana* showed a general decline from 7 – 14 DPI for all treatments (Fig. 4.1).

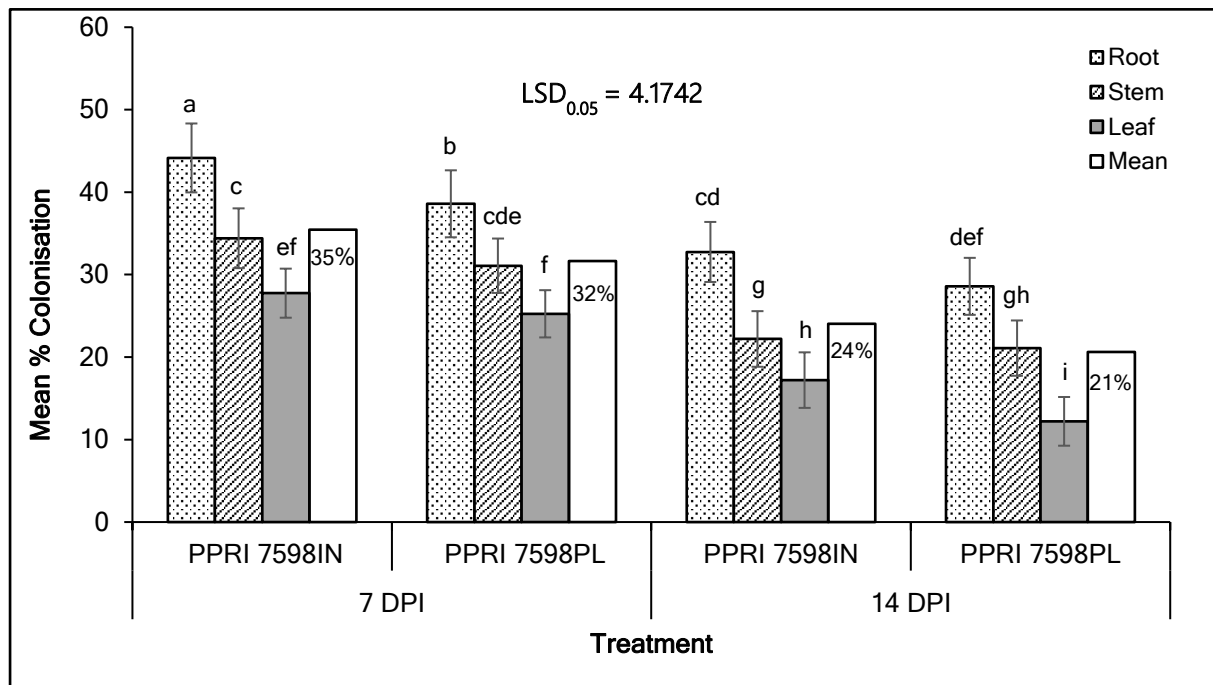


Figure 4.1 : Colonisation of different plant parts by *B. bassiana* from two different ‘backgrounds’ (PL vs IN) at 7 and 14 DPI. The bars (Means±SEM) marked with different letters indicate significant differences at the $P < 0.05$; LSD = 4.1742.

4.4 Discussion

Successful colonisation of roots, stems and/or leaves, following seed imbibition with *B. bassiana*, has been reported for wheat (Russo *et al.*, 2015; Sánchez-Rodríguez *et al.*, 2015; Sánchez-Rodríguez *et al.*, 2018), broad bean (Jaber and Enkerli, 2016), sorghum (albeit only in sterile soil; Tefera and Vidal, 2009) and also for cotton, tomato and snap bean, following topical seed inoculation with dry conidia (Ownley *et al.*, 2008). Similar results were found in our study, although compared with other plant parts, a significantly higher level of endophytism was observed in roots for 7598IN and 7598PL,

at both 7 and 14 DPI. Varying levels of endophytism demonstrate some degree of differential plant part colonisation, as reported in various studies (Tefera and Vidal, 2009; Gurulingappa *et al.*, 2010; Akutse *et al.*, 2013; Jaber and Enkerli, 2016). The differential plant part colonisation could presumably be an attribute of endophytic fungi adaptation within the variable plant parts (Petrini and Fisher, 1987). Clearly, uptake and systemic spread of *B. bassiana* (Behie *et al.*, 2015) via seed treatment, holds significant potential as an economical and practical inoculation method; especially for wheat, where seeding volumes range between 15 – 30 kg per hectare (ARC-Small Grain, 2018). Although wheat colonisation declined with time (7 – 14 DPI) in this study, the same trend has been observed also in other crops (Posada *et al.*, 2007; Gurulingappa *et al.*, 2010; Jaber, 2015). Although the current study did not measure endophytic persistence beyond 14 days, fungal colonisation of host plants at the early developmental stage may provide a crucial competitive advantage to these plants, potentially improving their ability to cope with stress and/or utilise limited resources (Hubbard *et al.*, 2013; Bokati *et al.*, 2016). In SA, wheat cultivated under dryland conditions in the summer rainfall region, is typically sown in the winter months of May-July and emerge during June-August, just prior to the arrival of spring rains in late September or early October, onwards. During and shortly after seedling emergence, soil moisture levels (residual from the preceding summer season) continue to decrease amidst increasing temperatures. Endophytic colonisation of such seedlings, with special emphasis on improved root development (on average, 65% increase in fresh root biomass among the three dryland-production cultivars) through possible indoleacetic

acid or auxin production (Liao *et al.* 2017) and/or improved Fe nutrition (Sánchez-Rodríguez *et al.* 2015), may hold significant advantages; a notion supported by the findings in our plant-response trials.

For all five plant growth parameters, *viz.*, shoot height, root length, fresh shoot biomass, fresh root biomass and dry shoot biomass, measured within 42-45 DPI, fungus-treated plants significantly outperformed control plants. A similar, positive growth response with *B. bassiana*-inoculated wheat, was reported by Gurulingappa *et al.* (2010) and more recently by Sánchez-Rodríguez *et al.* (2018), including an impressive 40% increase in wheat grain yield. Using only one wheat cultivar (Chinese Spring) and essentially the same techniques and conidial concentrations as in this study, Sánchez-Rodríguez *et al.* (2018) reported superior colonisation and subsequent growth responses with seed imbibition and soil drenching, compared to leaf spraying having little to no effect compared to controls. A similar 'cultivar-specific' reaction may be involved with cultivar Kariaga, showing a very high affinity towards seed treatment with *B. bassiana* 7598. Another interesting observation from the studies by Quesada-Moraga *et al.* (2014) and Sánchez-Rodríguez *et al.* (2018), is vertical transmission of *B. bassiana* within endophytically colonized maternal plants. This phenomenon could hold potential also for suppression of wheat seed - related diseases like Karnal bunt (KB; caused by *Tilletia indica* (Carris *et al.* 2006)) and *Fusarium* head blight (FHB; caused by anamorphic *Fusarium graminearum* (Dean *et al.*, 2012)), both not only present in SA, but globally. According to a study by Rabiey and Shaw (2016), application of the root-colonising

endophyte, *Piriformospora indica* (Sebacinaceae) to wheat, reduced FHB disease severity and incidence by 70%, while lowering mycotoxin (DON) concentration of winter and spring wheat samples by 70% and 80%, respectively. It also increased aboveground biomass, 1000-kernel weight and total grain weight. Although *Beauveria* is known to endophytically suppress damping-off in cotton (Griffin, 2007), bacterial blight in tomato (Ownley *et al.*, 2008), Zucchini yellow mosaic virus in squash (Jaber and Salem 2014) and downy mildew in grapevines (Jaber, 2015), its biocontrol potential against FHB is yet to be explored. In support of such notion, Gdanetz and Trail (2017) recently demonstrated the 'protective role' of the naturally occurring wheat microbiome (fungi and bacteria) against *F. graminearum*.

Artificial establishment of *B. bassiana* as an endophyte in wheat or any other host plant implies subjecting the fungus to a different growing environment in terms of nutrients, pH or phytochemicals. Generally, passage of an EPF through an insect host is seen as advantageous, *i.e.* a means of either enhancing or restoring virulence (Song and Feng, 2011). However, the effect on virulence following passaging through a plant has remained largely unexplored. As pointed out by Vidal and Jaber (2015), it is expected that antagonistic interactions between already established endophytes (note, axenic plants were not used in our study, although possible through embryo excision; see Robinson *et al.* 2016) and artificially introduced EPFs are the rule rather than the exception; potentially weakening the introduced endophyte (Kempf and Wolf 1989). Likewise, phytochemicals within the host plant may also pose antimicrobial activity

against EPF through antibiosis, as was hypothesized by Poprawski and Jones (2001), particularly in wheat (Rajoria *et al.*, 2015).

This study has underscored several positive aspects related to endophytic *B. bassiana* in wheat and contributed to our appreciation of the phenomenon, also being explored locally in crops like sugarcane (Memela, 2014; Memela *et al.*, 2017) and 'Rooibos' (Hatting, 2017), an indigenous shrub from which tea is made through leaf fermentation. Clearly, expanding the local use of *B. bassiana* (and other EPFs) from a topically applied bioinsecticide (Hatting *et al.*, 2018) to systemic bioinsecticide, also with disease suppressive and/or plant growth promoting attributes (in addition to wheat, see also Sasan and Bidochka, 2012; Lopez and Sword, 2015; Jaber and Enkerli, 2017), creates new and sustainable IPM possibilities, also supporting poverty alleviating and national food security.

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CHAPTER 5: ENDOPHYTIC EFFECT OF *BEAUVERIA BASSIANA* STRAIN PPRI 7598 AGAINST *DIURAPHIS NOXIA* RWASA1: MEASUREMENT OF APHID DEMOGRAPHIC GROWTH PARAMETERS ON SELECTED SOUTH AFRICAN *TRITICUM AESTIVUM* (L.) CULTIVARS

5.1 Introduction

Endophytes are commonly defined as microorganisms of fungal or bacterial genera which inhabit asymptomatic plant tissue for all or part of their life cycle without inflicting detectable damage (such as lesions, poor growth, discoloration or chlorosis to the host (Petrini, 1991; Hardoim *et al.*, 2015; Puri *et al.*, 2016). In this study, we hypothesise that endophytes can be inoculated for endophytic colonisation of a wide range of crops to evaluate their effect on insect herbivores (Porrás-Alfaro and Bayman, 2011).

Effects of endophytic plant colonisation were mostly investigated from clavicipitaceous fungal endophytes that are vertically transmitted through seeds and systemically proliferate within above ground parts of grasses (Jaber and Vidal, 2010). The method of inoculation is the fundamental part to the reported positive effects of endophytes on vertically colonised plants. Quesada-Moraga *et al.* (2014) reported the success of vertical transmission of *B. bassiana* through seed treatment for control of insect herbivores. Mutualistic endophytes were found to be ubiquitous associates of most plant species and indirectly secure plant defence against herbivores (Hartley and Gange, 2009). By production of toxic substances (alkaloids) and metabolites within the plant

tissue, fungal endophytes can inhibit insect feeding (Kim *et al.*, 2004; Quesada-Moraga *et al.*, 2006) and kill insect herbivores at their feeding site (Rohlf and Churchill, 2011; War *et al.*, 2012). In various studies, clavicipitaceous endophyte colonisation of ryegrass leaves deterred the aphid from feeding, thereby negatively affecting the aphid's reproduction relative to that on endophyte-free plants (Wilson *et al.*, 1991; Gurulingappa *et al.*, 2010; Rondot and Reineke, 2018). This mechanism, which forms the hypothesis of this study, is understood to be attributed to the ability of the endophyte to alter host plant nutritional quality (Bernays, 1993) and secrete secondary metabolites (Arnold, 2008) and volatiles (Jallow *et al.*, 2008), which reduces the insect feeding urge on endophyte-colonised plants.

Interestingly, most studies confirmed successful reduction of insect herbivory from piercing-sucking, boring, chewing and leaf-mining pests residing on endophyte-colonised plants (Bing and Lewis, 1991; Jallow *et al.*, 2008; Vega, 2008). Among the sucking insects, whiteflies and aphids are wide spread. Aphids are most notorious and capable of causing a considerable damage on cereal crops, contributing to yield loss (Agarwal and Datta, 1999). The Russian wheat aphid (RWA), *Diuraphis noxia* (Hemiptera: Aphididae) is one of the main pests of small grain cereal crops, including bread wheat, *Triticum aestivum* L. In the current study, we evaluated the systemic effect of endophytic *B. bassiana*, strain PPRI 7598, on *D. noxia* biotype (RWASA1) on three SA *T. aestivum* cultivars. The objectives of this study were: a) to determine the effect of the endophyte on RWA reproduction and population growth and b) to assess the effect of the

endophyte on aphid biomass and *T. aestivum* response towards *D. noxia* herbivory using a damage rating index (Tolmay, 1995).

5.2 Materials and methods

5.2.1 Experimental design

In experiment 1, a series of three independent trials was conducted under glasshouse conditions. For each trial, the experimental design was a randomised complete block with 10 replicates (blocks). The treatment design was a split plot n. The main plot was three cultivars (Molopo, Gariep and Kariega) and the subplot was *B. bassiana* inoculated plants (treated) designated as "1" and untreated control plants as "0". Sixty plants (10 plants x 2 treatments x 3 cultivars) per trial were assayed against *D. noxia* infestation.

In experiment 2, three sets of trials were conducted. For each trial, the experimental design was a randomised block with three replicates (blocks). The treatment design consisted of two factors, cultivars with three levels (Molopo, Gariep and Kariega) and *B. bassiana* treatment with two levels (treated and untreated). The experiment consisted of 90 plants (3 cultivars x 5 replicates x 3 trials x 2 treatments).

5.2.2 *Aphid rearing*

The RWASA1 colonies were reared in the ARC-Small Grain insectary unit. An aphid clone was established from one adult female which was maintained on a single intact plant for 24 hours in the laboratory at room temperature. The adult aphid was then removed, leaving a clone of first filial generation (F1) nymphs on the plant. Each of the F1 generation nymphs in the first instar was transferred to a single wheat seedling at (Jankielsohn, 2013), reared in a gauze-covered cage in a glasshouse at $22 \pm 3^{\circ}\text{C}$, $70 \pm 4\%$ relative humidity (RH), natural light. Aphids were monitored until they reached the adult reproductive stage and produced the F2 generation for use in the trial. F2 generation aphids were further monitored for one week before they were transferred to experimental and control plants, also at the four-leaf growth stage using a fine camel hair brush.

5.2.3 *Endophytic fungal strain*

The *B. bassiana* fungal strain PPRI 7598, shown to be virulent against the RWA in chapter 3 and potentially colonising wheat seedlings following seed treatment in Chapter 4, was again used in this study. The fungal strain was cultured on *B. bassiana* selective medium amended with 0.55g/L dodine (guanidine) and 0.005g/L chlortetracycline (Sigma – Aldrich, Germany) (Chase *et al.*, 1986). Fungal cultures were incubated in full darkness at $25 \pm 1^{\circ}\text{C}$ and $60 \pm 10\%$ relative humidity. Conidia were harvested from 14 days old cultures using a sterile scalpel and suspended in a sterile

aliquot of distilled water with 0.01% Break Thru[®] surfactant (Polyether-polymethylsiloxane-copolymer surfactant; Goldschmidt Chemical Corporation, USA). Fungal suspension was then vortexed for at least 5 minutes to obtain a homogenous stock suspension. Suspension concentration was adjusted to 10^8 conidia ml⁻¹ using the Nikon Optiphot light microscope (Nikon, Japan) and improved Neubauer, 0.1 mm depth Haemocytometer (Neubauer, Germany).

5.2.4 *Wheat seed inoculation and planting*

Seeds of three SA *T. aestivum* cultivars (Molopo, Gariep and Kariaga) were obtained from the ARC- Small Grain Germplasm collection. Seed were surface sterilised based on Akello and Sikora (2012) procedure (with minor modifications). Following sterilisation, seeds were dried in the laminar flow for 30 minutes and later immersed in a *B. bassiana* fungal suspension concentration of 1×10^8 conidia ml⁻¹. Control seeds were soaked in 5ml of sterile distilled water with 0.01% Break Thru[®] surfactant. Twenty seeds were soaked for 18-24 hours in 5 ml spore suspension with occasional aeration while maintained on an orbital shaker at room temperature (Dhingra and Sinclair, 1995). Inoculated and control seeds were later dried on sterile paper towel in the laminar flow for three hours prior to planting. Seeds were first plated between moist filter papers (Lasec, SA) in 90 mm petri dishes, incubated in the dark at $25 \pm 1^\circ\text{C}$ to enhance germination. Seedlings were later transplanted singly in cones (100 ml) which contained sterile soil/sand substrate (3:1 v/v), sterilised in the autoclave at 121°C for 15 minutes.

Plants were maintained in water - filled trays under greenhouse conditions ($22 \pm 3^{\circ}\text{C}$, $70 \pm 4\%$ relative humidity (RH)) under natural light for 21 days. Sixty experimental plants (3 cultivars x 10 replicates x 2 treatments) each were later infested with one of the age synchronised RWA adults (8 days old).

5.2.5 In vivo bioassays

5.2.5.1 Experiment 1: Effect of the endophyte on the RWA reproduction and population growth with intrinsic rate of increase

The RWA colony/clone was established in the greenhouse under similar conditions as above (section 5.2.2). Experimental design as explained in section 5.2.1. The same greenhouse conditions were applicable to experimental (1) and control (0) plants. The experiments were performed on un-infested intact plants. Separate trials consisted of sixty individual plants (four leaf growth stage), each infested with one F2 generation aphid at reproductive adult stage (8 days old). All plants with aphids were enclosed in clear ventilated plastic bottles (16 cm x 9 cm), inverted to cover plants and prevent aphids from escaping or mixing between treatments. Aphid reproduction based on number of born nymphs was recorded daily from each plant for a 15-day period. Following enumeration, all newly born nymphs were removed from plant leaves while adults remained. Net reproductive rate (R_0) and mean generation time (T) were estimated across three cultivars and treatments to determine the intrinsic rate of aphid

population increase (r_m) which was calculated based on the following formula (Birch, 1948):

$$\sum e^{-rx} (l_x \times m_x) = 1$$

Where x is the time increment (24h), l_x is the probability of being alive on day x , m_x is the average birth rate on day x and r is the intrinsic rate of increase. Experiments were repeated thrice, 7 days apart.

*5.2.5.2 Experiment 2: Effect of the endophyte on aphid biomass and *T. aestivum* response towards RWA herbivory*

Systemic effect of plant endophytic colonisation was evaluated from three wheat cultivars maintained under greenhouse conditions of 22°C (day) and 15°C (night) with natural light. There were 30 plants per cultivar with two treatment levels per batch (endophyte treated and untreated). All 90 plants (3 cultivars x 5 replicates x 3 blocks x 2 treatments) were organised in a completely randomised design on a 94-cone seed tray/flat placed in water-filled trays. A tray with all plants at the 4-leaf growth stage was infested with 450 apterous *D. noxia*, weighing 0.05016375 g (mixed instars taken from the greenhouse colony), giving an average infestation of five aphids per plant (Tolmay *et al.*, 1999). Plants were evaluated for their response towards RWA herbivory and scored for *D. noxia* damage three weeks (21 days) after infestation. Severity of infestation was evaluated based on damage rating levels from 1-10 (Table. 5.1) per plant (Tolmay, 1995).

Table 5.1: Description of Russian wheat aphid damage symptoms used for scoring.

Adapted from Tolmay (1995).

Rating	Description of symptoms
1	Small isolated chlorotic spots
2	Small chlorotic spots
3	Chlorotic spots in rows
4	Chlorotic splotches
5	Mild chlorotic streaks
6	Prominent chlorotic streaks
7	Severe streaks, leaves fold conduplicate
8	Severe streaks, leaves roll convolute
9	Severe streaks, leaves roll tightly
10	Plant dying

This index was deployed to assess the feeding intensity and damage levels caused by RWA herbivory on both treated and control plants. Determination of population growth was performed at day 22 of aphid infestation.

5.2.5.3 *Confirmation of plant endophytic colonisation*

Endophytic colonisation of wheat plants was determined at 6 weeks post inoculation i.e at the end of experiment 1. Two detection methods based on culturing (fungal re-isolation) and species-specific PCR diagnostic techniques were deployed to confirm *B. bassiana* endophytic colonisation of wheat seedlings across three selected cultivars. Upon termination of each trial all 60 experimental plants (treated and control) were harvested. Adapted from Castillo-Lopez *et al.* (2014), two leaves from each plant were surface sterilised (Bills, 1996) and excised into two longitudinal halves using a sterile

scalpel. One half was fragmented to 1 cm lengths and plated on *B. bassiana* selective medium (as per 5.2.3). Unlike with the previous chapter, evaluation for endophytic colonisation of seedlings was focused on leaves only (RWA feeding site). Cultures were evaluated for fungal growth from day 7 – day 10 of incubation. Colonisation rate was calculated as a number of colonised segments divided by total number of segments examined, multiplied by 100 (Fisher and Petrini, 1987). However, data were only captured to confirm positive endophytic colonisation of plants and not for statistical analysis.

The other half of each leaf was freeze-dried using a freeze dryer (Christ® Alpha 2-4 LD plus, Mechatechsystems, UK) and DNA was extracted using the CTAB protocol (Doyle and Doyle, 1987). Diagnostic PCR analysis was performed using *B. bassiana* species-specific primers (Castillo-Lopez *et al.*, 2014) to amplify the endophyte DNA. PCR amplicon was visualised on 1.5% agarose gel using gel electrophoresis under UV illuminator (Molecular Imager® Gel Doc™ XR System, Bio-Rad, SA). Fragment size of the amplified DNA from inoculated endophyte was confirmed based on the positive control strain (PPRI 7598) size.

5.3 Statistical analysis

The statistical procedure performed was similar to those described in chapters three and four. Homogeneity of trial variances was verified by Levene's test (Levene, 1960).

The normality of the standardized residuals was confirmed using the Shapiro-Wilk test (Shapiro and Wilk, 1965). The data of the combined trials were subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Repeated measurements of the data over days were combined as a subplot factor in a split plot analysis of variance (Little and Hills, 1978). Fisher's protected least significant difference (LSD) was calculated at the 5% level to compare treatment means (Ott and Longnecker, 2001). A probability level of 5% was considered significant for all significance tests.

5.4 Results

5.4.1 Effect of the endophyte on the RWA reproduction and intrinsic rate of population increase

Significant effects were observed between the two plant treatments, with versus without endophyte (endophyte+ and endophyte-, respectively) at $P < 0.05$ (Table 5.2a). The effect of the two treatments varied significantly in terms of net reproductive rate (R_0) and the intrinsic rate of increase (r_m) of the RWA population over the average of 6.5 days' mean generation time (T) across the three trials (Table 5.2a).

Table 5.2a: Representation of different life table parameters that influenced *D. noxia* intrinsic rate of increase, r_m under different trials and treatments.

Trials	Life table parameters		
	Net reproductive rate (R_0)	Mean generation time (T)	Intrinsic rate of increase (r_m)
1	29.65±0.32a ¹ (1) ²	6.0542±0.47b (3)	0.560±0.13a (1)
2	24.52±0.28b (2)	6.2018±0.59b (2)	0.504±0.15b (2)
3	21.37±0.41b (3)	7.3061±0.53a (1)	0.417±0.16c (3)
Grand mean	25.18±2.41	6.5207±0.39	0.494±0.04
LSD _{0.05}	3.592	0.5171	2.0049
Treatment	R_0	T	r_m
- Endophyte (0)	31.39±0.24a (1)	6.759±0.42a (1)	0.517±0.12a (1)
+ Endophyte (1)	17.21±0.33b (2)	6.282±0.69b (2)	0.471±0.20b (2)
Grand mean	24.3±7.09	6.521±0.24	0.494±0.02
LSD _{0.05}	1.8293	0.3824	0.0282

¹Means±SEM within columns followed by the same letter are not significantly different at the 5% test level

² Performance ranking within a given life table parameter (column) in parenthesis

A significant difference in RWA population growth (R_0 and r_m) was observed between trials ($P < 0.05$; LSD 0.6401 and $P < 0.05$; LSD 0.0488, respectively), resulting in the lowest intrinsic rate of 0.372 ±0.022 nymphs female⁻¹ d⁻¹ with endophyte treated plants compared to 0.462 ±0.010 for the control in trial 3 (Table 5.2b). A negative effect of the treatment on the RWA R_0 was noted on endophyte treated plants, reducing the population growth by 14 nymphs female⁻¹ d⁻¹ compared to the plants without the endophyte (control) (Table 5.2b). However, the results indicated a poor correlation between the R_0 and r_m across trials, although treatments were significant at $P < 0.0001$. Overall, the endophyte significantly reduced the RWA population in treated plants over the control (Table 5.2b).

Table 5.2b: The effect of the treatment (with versus without the endophyte) on *D. noxia* population increase in different trials.

Trial	Reproduction rate (R_0)		Intrinsic rate (r_m)	
	+ Endophyte	- Endophyte	+ Endophyte	- Endophyte
1	22.33±0.269c ¹ (1) ²	36.97±0.230a (1)	0.557±0.023a (1)	0.563±0.012a (1)
2	17.67±0.182d (2)	31.37±0.240b (2)	0.483±0.020bc (2)	0.526±0.018ab (2)
3	14.33±0.229d (3)	28.400±0.201b (3)	0.372±0.022d (3)	0.462±0.010c (3)
*G. mean	18.11±2.32	32.25±2.51	0.471±0.05	0.517±0.03
LSD _{0.05}	0.6401		0.0488	

¹ Means±SEM within columns followed by the same letter are not significantly different at the 5% test level

² Performance ranking within a given life table parameter (column) in parenthesis

*Grand mean

The results showed that there were no interactions between trials and cultivars. However, an interaction between cultivars and treatment was noted. The cultivar response towards the treatment influenced the RWA population increase significantly ($P<0.05$). Significant effect of the endophyte on RWA settlement on different cultivars was noted at $P<0.05$ level, with the endophyte inducing a significant of *D. noxia* R_0 by approximately 14 nymphs female⁻¹ ($P<0.05$) in all cultivars (Table 5.3). While Kariaga and Molopo are known to be susceptible to *D. noxia* RWASA1, the presence of the endophyte induced some level of resistance in Molopo towards *D. noxia*. Such resistance impacted aphid R_0 levels negatively on Molopo similarly as in Gariep (resistant cultivar). This indicated that Kariaga remained susceptible to *D. noxia* even in the presence of the endophyte, hence it supported the aphid reproduction to an average of 27.39 ±0.36 (Table 5.3).

Table 5.3: The effect of the treatment (with and without the endophyte) on *D. noxia* population increase across cultivars.

Cultivars	Reproduction rate (R_0)		
	+ Endophyte	- Endophyte	Average
Kariega	20.64±0.3c ¹ (1) ²	34.15±0.268a (1)	27.39±0.36a (1)
Molopo	16.65±0.234d (2)	31.11±0.263ab (2)	23.88±0.37b (2)
Gariep	16.34±0.167d (3)	28.92±0.191b (3)	21.63±0.33b (3)
*G. mean	17.88±1.38	31.39±1.52	24.3±1.68
LSD _{0.05}		3.168	3.1437

¹Means±SEM within columns followed by the same letter are not significantly different at the 5% test level

²Performance ranking within a given life table parameter (column) in parenthesis. * G. mean denotes the Grand Mean.

5.4.2 Effect of the endophyte on aphid mass and *T. aestivum* response towards RWA herbivory

With the assumption that the total initial mass of aphids (0.05016375g) halved to equally infest both groups of endophyte treated and control plants with 0.025081875g, the aphid masses differed significantly ($P < 0.05$) between the treatments based on suitability of plants for foraging. Significantly higher aphid masses ($P < 0.05$) were observed on endophyte free plants, the highest on RWASA1 susceptible cultivars than on resistant Gariep. While the mean aphid mass remained the same on Kariega endophyte- and Kariega endophyte+, the other two cultivars recorded the similar mass values in the presence of the endophyte only. Thus, the endophyte had a significant effect on *D. noxia* mean mass in Molopo by 43% and 29% in Gariep compared with the endophyte free plants (Fig. 5.1a).

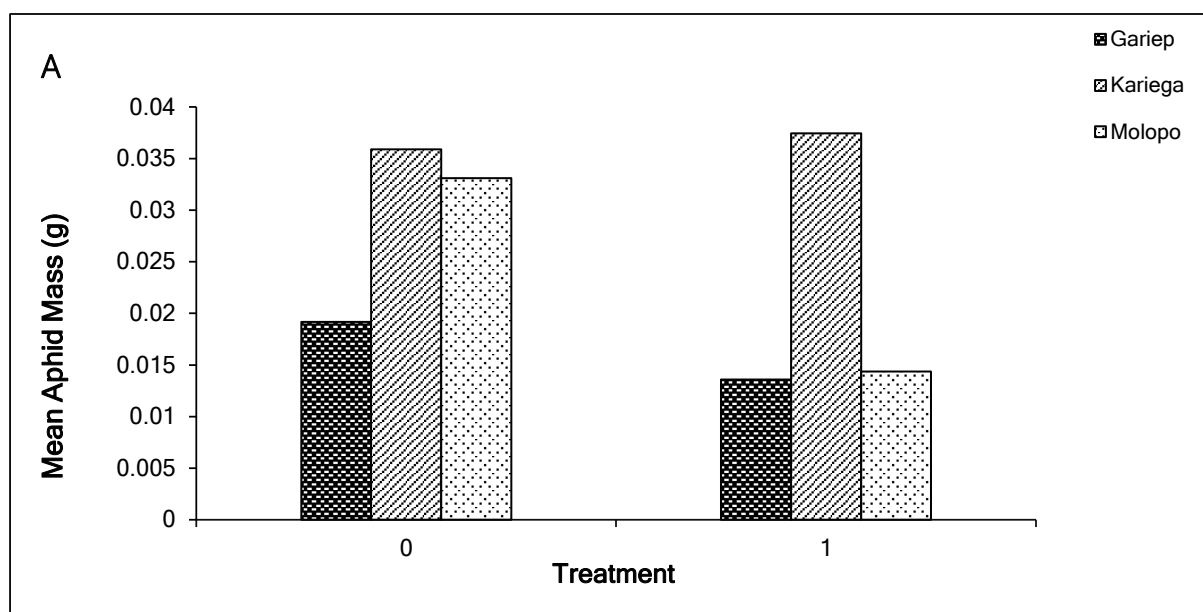


Figure 5.1a: Distribution of the mean aphid mass at the end of the experiment. The indices 0 and 1 denoted: 0 = treatment without the endophyte (Endophyte-) and 1 = treatment with an endophyte (Endophyte+). Total initial aphid mass was calculated as: 90 plants x 5 aphids x RWASA1 mass (0.000111475) = 0.05016375g.

Overall, the mean aphid mass increased by 17% from the initial 0.025081875 g in control plants to the final mass of 0.029392g; whereas the endophyte presence significantly decreased the aphid mass by 13% over the controls, all cultivars pooled ($P < 0.05$; $LSD = 0.0065$) (Fig.5.1b).

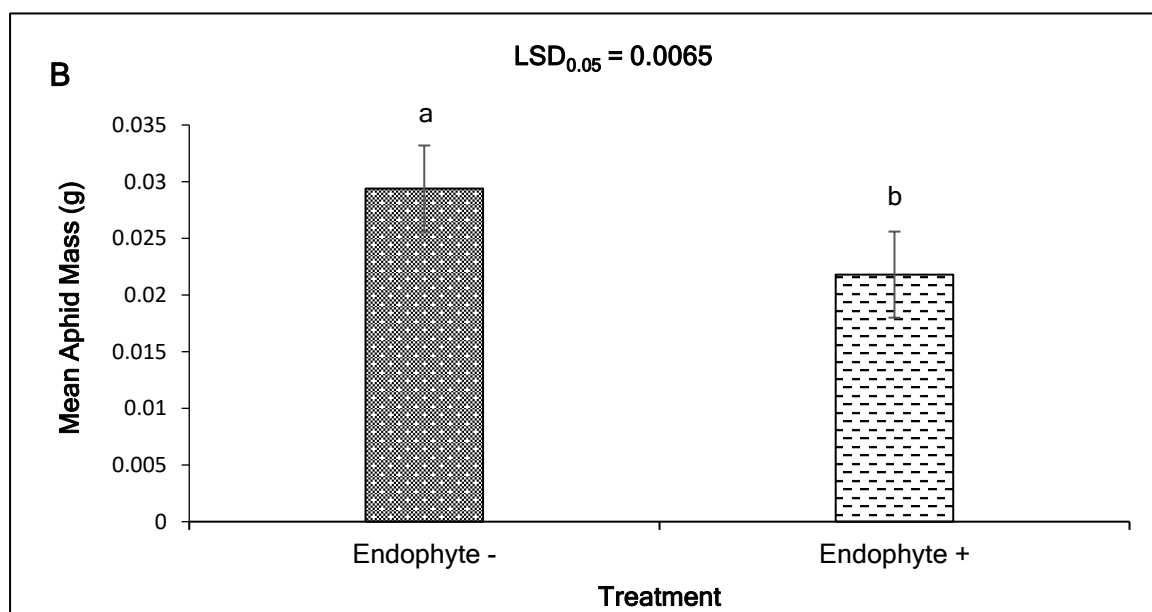


Figure 5.1b: The mean aphid mass for the endophyte treated and control plants, all cultivars pooled. Bars (Means±SEM) marked with different letters indicate significant differences at the $P < 0.05$; LSD = 0.0065.

However, the damage rating varied significantly ($P < 0.05$) across the three cultivars, the highest in Molopo (9) followed by Kariaga and Gariep (8 & 5, respectively) (Fig. 5.2), Gariep being the only RWASA1 resistant cultivar, although there was no significant difference between the treatment levels across the three cultivars at $P \leq 0.0001$.

Category	Scoring scale	Response
Resistant	1 = Small isolated chlorotic spots 2 = Small chlorotic spots 3 = Chlorotic spots in rows	
Moderately resistant	4 = Chlorotic splotches 5 = Mild chlorotic streaks	Gariep
Moderately susceptible	6 = Prominent chlorotic streaks 7 = Severe streaks, leaves fold conduplicate	
Susceptible	8 = Severe streaks, leaves roll convolute 9 = Severe streaks, leaves roll tightly 10 = Plant dying	Kariega Molopo

Figure 5.2: Damage rating analysis for the three cultivars induced by *D. noxia*, RWASA1. Adapted from Tolmay, (1995).

5.4.3 Confirmation of plant endophytic colonisation

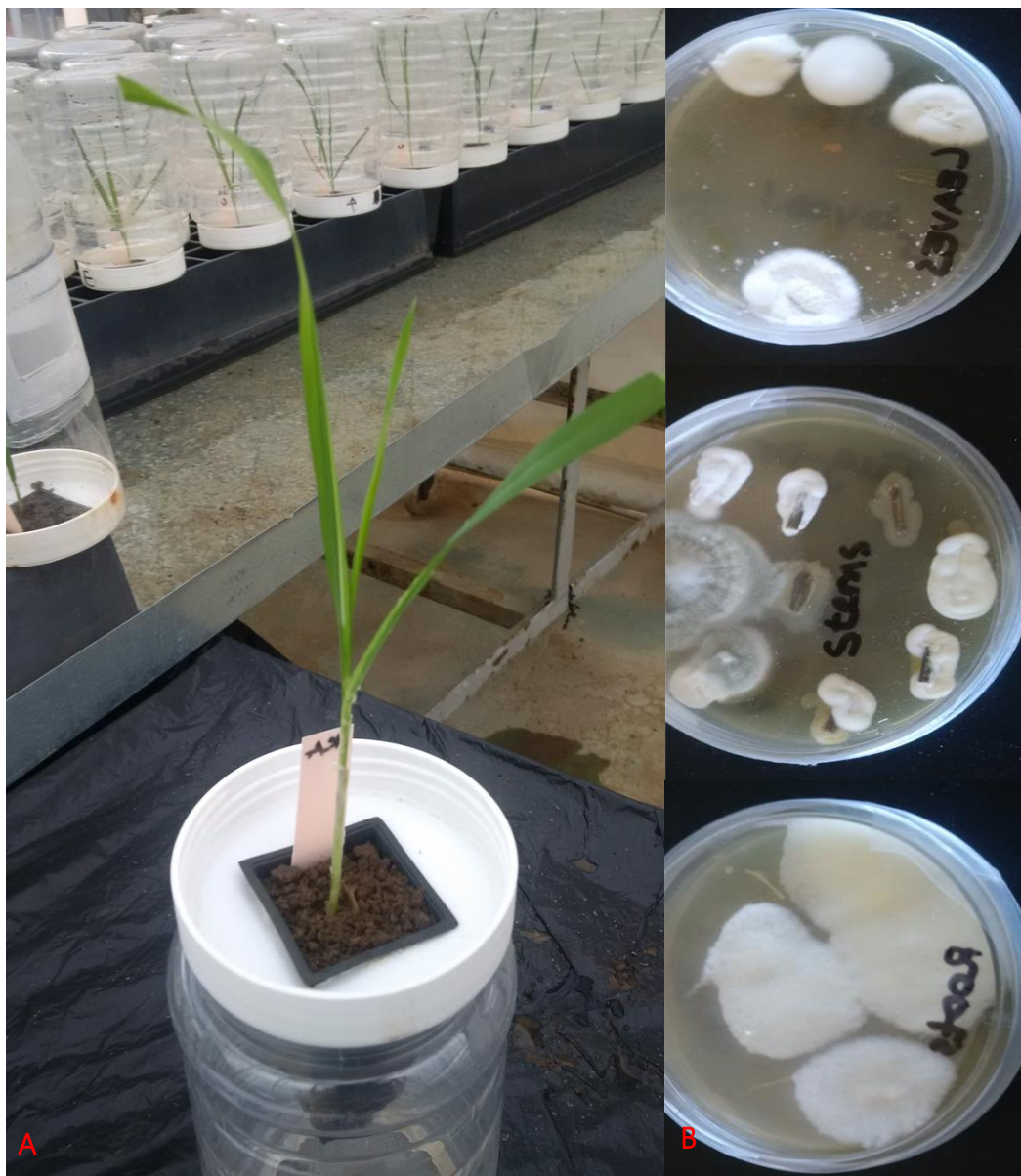


Figure 5.3: (A) Endophyte inoculated plants planted singly in the cone. Plants were maintained under glasshouse conditions for 15 days after infestation of each plant with one adult aphid (*D. noxia*). (B) Evaluation of plant colonisation from different sections of leaves, stems and roots. Isolation of endophytic fungus as observed on colonised plant sections.

Physical recovery of the endophytic strain from the inoculated plants was performed using *B. bassiana* selective medium (colonisation data not shown). Although

colonisation and isolation success were very low in most plates, the observed fungal growth shows the presence of the fungal endophyte in different plant tissues. Confirmation of endophytic colonisation of wheat seedlings of different cultivars was covered in full in chapter 4.

5.5 Discussion

In the present study, the effect of endophytic *Beauveria bassiana* strain PPRI 7598 on *D. noxia* was tested for the first time in SA. However, there is currently a limited knowledge available to support the endophytic colonisation effect on the *D. noxia* herbivory on SA wheat cultivars. While *D. noxia* was maintained on three *T. aestivum* cultivars, population growth (with- and without endophyte) was evaluated from different life table parameters. Intrinsic rate of natural increase (r_m) of *D. noxia* population was estimated from the net reproductive rate (R_0), and mean generation time (T). The intrinsic rate of increase (r_m) can be interpreted as the number of births minus the number of deaths per generation time and calculated as the natural logarithm of the net reproductive rate divided by the mean generation time ($r = \ln R_0 / T$) (Ricklefs, 1993). Values above zero indicate that the population is increasing. The higher the value of r_m , the faster the intrinsic rate of the population increase (Ricklefs, 1993). The net reproductive rate (R_0) which forms part of r_m denotes the average number of offspring the female produces during her lifetime ($R_0 = \sum l_x m_x$). While R_0 of 1.0 indicates population stability, a rate above 1.0 indicates an increase in population (Grant and Grant, 1992).

The evidence of increasing population rates of aphid with R_0 values above 1.0 were observed across all the three trials and cultivars. Significantly higher R_0 rates were noted in trial 1 both with- and without the endophyte whereas the r_m was only significant ($P < 0.05$) in trial three. However, the presence of an endophytic *B. bassiana* negatively affected *D. noxia* reproduction on inoculated plants (three cultivars pooled) compared with control plants in a series of three individual trials. Similar results were also reported on cotton aphids by Gurulingappa *et al.* (2010) and Castillo-Lopez *et al.* (2014). As highlighted by Martinuz *et al.* (2012) and Akello and Sikora (2012) in similar studies, poor aphid performance could be an attribute of chemical changes (toxic alkaloids) induced by the endophyte presence in host plants. Although r_m did not differ significantly ($P \leq 0.0001$) across all tested cultivars, the highest intrinsic rate (suggesting rapid intrinsic rate) recorded in trial 3 could have been due to the highest settlement of *D. noxia* on Karioga relative to the other two cultivars. However, these results demonstrated a poor correlation between R_0 and r_m although treatments were significant at $p \leq 0.0001$.

Following the previous study conducted in SA by Jankielsohn (2013), among the three aphid biotypes (RWASA1, RWASA2 and RWASA3) tested, RWASA1 showed a positive intrinsic rate of increase on different cereal host plants. Although the endophyte was not involved in this investigation, RWASA1 intrinsic rate of population increase was higher in the susceptible cultivar (Tugela) compared with the resistant Tugela *Dn*. In line with these results, RWASA1 demonstrated a similar trend by showing the highest

intrinsic rate for Karioga over Gariep, even in the presence of the endophyte ($P < 0.05$; $LSD = 3.168$), indicating varying levels of host plant suitability for this aphid.

In the current study, an increasing *D. noxia* population was apparent on Karioga suggesting that this cultivar might be the most suitable host. Consequently, this resulted in increased *D. noxia* R_0 value (27.39 ± 0.36), coupled with the highest aphid masses (although not significant at $P < 0.05$) with- and without the endophyte (0.0374g and 0.0359g, respectively) as compared to the other two cultivars. This indicates that the endophyte metabolites could not alter this cultivar's palatability and aphid feeding preference. High plant palatability to sucking insects has been investigated in various studies (Bolser and Hay, 1996; Siska *et al.*, 2002; Pennings *et al.*, 2009) and was found to result in high herbivore population densities (Ho and Pennings 2013). This notion supports the findings in the current study. More plant palatability supports increased herbivore growth and feeding pressure due to weaker defence against herbivores, particularly at high versus low latitudes (Ho and Pennings, 2013) i.e. presumably in cold winter regions. Based on this fact, the geographic location of Bethlehem (Free State) (winter wheat production area), which is at an elevation of 1636 m above sea level, the preference of Karioga by RWASA1 over the other two cultivars could also be associated with the altitude.

Several studies performed on cereal aphids reared on different wheat cultivars indicated that decreased performance and reproduction are related to variable concentrations of allelochemicals such as Hydroxamic acids (Hx), phenolic compounds and indole

alkaloids (Argandoña *et al.*, 1982; Leszczynski and Dixon, 1990; Givovich and Niemeyer, 1995). Hx are wheat secondary metabolites which confer cereal resistance against aphids (Niemeyer and Pérez, 1995) and are known to prolong aphid development and reduce fecundity as well as the intrinsic rate of the natural increase (Dixon, 1987). In this case, relatively varying aphid populations found in Gariep (resistant) with (0.0136 g) and without endophyte (0.0192 g) suggest that both the Hx and the endophyte secondary metabolites had an effect to deter feeding in *D. noxia*. Production of alkaloids by the endophytes increases host plant competitive ability and resistance to biotic and abiotic stressors (Kuldau and Bacon, 2008) particularly insect herbivory. In support of this point, the evidence of endophyte-mediated negative effects on herbivore's foraging was confirmed with the resistant cultivar, Gariep in this study. Therefore, an integration of both the plant and endophyte defence mechanisms improved plant resistance, significantly lowering the aphid masses on endophyte treated plants by 26% ($P < 0.05$). However, the parity of the aphid mass between Molopo (susceptible) and Gariep in the presence of the endophyte was observed. This could indicate that the endophyte had potentially improved the host resistance towards aphid herbivory in Molopo.

Resistance of the host plant against insect herbivory buffers against adverse conditions associated with pest damage. A distinctive damage rate by *D. noxia* (RWASA1) between the resistant wheat cultivar (Gariep) and susceptible cultivars Molopo and Kariaga in the presence of the endophyte was recorded in this study. The level of damage in these cultivars varied significantly ranging from chlorotic leaf streaking to leaf rolling or

manifesting both depending on the degree of plant susceptibility and damage caused (Tolmay, 1995), similarly as reported by Burd and Burton (1992). Leaf rolling reduces the host plant photosynthetic area while affording an optimum environment for aphid reproduction in the leaf whorl (Gutsche *et al.*, 2009). This condition was illustrated in this study when Molopo and Kariaga recorded the highest damage scores (9 & 8, respectively) with high aphid populations wrapped inside rolled leaves. The assumption around the methodology used to measure the plant damage was that aphids would be distributed evenly giving the average of five aphids per plant (Tolmay, 1995). However, the random distribution of aphids of mixed instars around plants held various possibilities for plant infestation. This arrangement might have afforded aphids a wider choice to select plants according to their preference towards susceptible plants, possibly avoiding settlement on resistant plants. In this case, based on preference, it could be assumed that majority of adult aphids started producing progeny in the beginning of the experiment, rapidly increasing the aphid mass on susceptible plants. Alternatively, immature aphids on endophyte treated plants might have stopped feeding, resulting in delayed reproduction compared with those on control (endophyte free) plants. A series of studies presented several mechanisms through which endophytic fungi reduce insect herbivore damage as reduction in the insect developmental rate (Akello and Sikora, 2012; Akutse *et al.*, 2013), causing feeding deterrence (McGee, 2002; Vega, 2008), retardation of insect growth, reducing survival and oviposition (Lacey and Neven, 2006; Martinuz *et al.*, 2012). Consequently, the aphid feeding characterised by secretion of saliva in the plant phloem contents could have

exacerbated plant damage by changing the host plant physiology as reported by Prado and Tjallingii (1994).

In majority of studies, physiological responses of resistant and susceptible cereals, particularly wheat towards *D. noxia* feeding was reported (Burd and Elliot, 1996; Franzen *et al.*, 2007; Macedo *et al.*, 2009). Research about such responses focused on chlorophyll and protein content, chlorophyll fluorescence, gas exchange and molecular pathways (Gutsche *et al.*, 2009). While in the study by Ni *et al.* (2002) susceptible plants encountered an alteration in chlorophyll contents with observable leaf chlorosis relative to resistant plants, the same scenario was confirmed in the current study. These observations are supported by the fact that *D. noxia* can alter the amino acid profile of the phloem contents which constitute the host plant nutrient alteration in susceptible wheat cultivars to its benefit (Telang *et al.*, 1999). Consequently, this phenomenon resulted in the retarded growth pattern observed in susceptible wheat cultivars in this study. This remarkable characteristic discovered in *D. noxia* could have supposedly contributed to significant damage levels recorded on Molopo albeit the reduced aphid mass response observed in our findings. However, the effect of *D. noxia* feeding had presumably failed to alter nutrient profile within the tested resistant cultivar (Telang *et al.*, 1999) Gariep, hence this cultivar succumbed to the aphid damage.

While most experiments evaluating the endophyte-insect interactions similar to the study by Gurulingappa *et al.* (2010) were performed on cut leaf bioassays, possible plant nutrient alteration caused by abscission of plant leaves is inevitable (Price *et al.*, 2011).

However, this study is one of the rare adventures conducted on *B. bassiana* inoculated intact plants (seed treatment). This exercise safeguards minimal plant chemistry changes emanating from leaf cutting, which could alter the interaction between the insect and the endophyte (Price *et al.*, 2011).

Nevertheless, mechanisms underpinning the endophyte-mediated plant nutritional quality and production of secondary metabolites are known to impact the insect feeding on the endophyte-colonised plants (Arnold, 2008). Although reported with *Rhopalosiphum padi*, resistant wheat plants may perhaps trigger some antibiosis-based responses against the aphid, by deploying some mechanisms that inhibit aphid induction of defensive compounds in wheat seedlings (Gianoli and Niemeyer, 1998). Remarkably, with a possible overlap with *D. noxia*, both the host plant resistance and endophytic induced systemic resistance could work to SA's advantage that RWASA1 may not adapt antibiosis and /or antixenosis responses in Gariep to break this cultivar's resistance. These observations could explain the significant difference in damage rates observed between susceptible and resistant wheat cultivars in this study.

Although colonisation data was not shown, endophytic colonisation of plants was relatively lower as compared with the results from the previous chapter. In all experiments or trials conducted in this study, endophytic colonisation was mostly observed on surface sterilised roots than on leaves (aphid feeding sites). Endophytic colonisation of inoculated plants was confirmed with the cultural isolation method using *B. bassiana* selective medium (Chase *et al.*, 1986). Although this trend was also reported

in Chapter 4, this could be attributed to low systemic transmission of the endophyte through the stem to the leaves.

Alternatively, the use of the surface sterilisation procedure intended to clean plants parts from resident epiphytes, might have under-estimated the colonisation rates owing to diffusion of chemicals into the plant parts (Ownley *et al.*, 2008; Lohse *et al.*, 2015), particularly leaves. As the two scenarios may account for the low colonisation results in this study, we also sought to agree with McKinnon *et al.* (2014) that the surface sterilisation method used could affect the sterilised material prior to culture-based and molecular valuation of the endophytic establishment. Additionally, since cutting of leaves (during evaluation) subjects a plant to damage, the subsequent allelochemical production around lacerated parts including leaves could obscure the existing plant endophytes (Price *et al.*, 2011), thereby resulting in poor detection of the target endophyte.

The findings of the current study have demonstrated the endophytic potential of *B. bassiana* strain, PPRI 7598 for control of *D. noxia* populations in RWASA1 susceptible cultivars (Molopo and Kariaga). Although the endophyte did not reduce plant damage by *D. noxia* in susceptible cultivars, the endophyte could potentially reduce herbivory damage on resistant cultivar Gariep. These results suggested a new possibility for compatibility of the host plant resistance and the endophytic induced systemic resistance in RWASA1 resistant wheat cultivars. However molecular verification of the

endophyte within plant tissues using PCR assays was not successful using the protocol used by Castillo-Lopez *et al.* (2014).

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CHAPTER 6: ISOLATION AND MOLECULAR DETECTION OF INDIGENOUS *BEAUVERIA BASSIANA* ISOLATES FROM SELECTED SOUTH AFRICAN LOCALITIES

6.1 Introduction

Beauveria bassiana (Bals.) Vuill. (Ascomycota: Hypocreales) is a globally distributed soil-borne hyphomycete of economic significance as model system in insect pathogenesis studies for its potential as mycopesticide (Rehner and Buckley, 2005). Isolation and detection of the EPF *B. bassiana* from different substrates in the environment are fundamental aspects that contribute prerequisite knowledge to address fungal biology and ecology. General detection and classification of EPFs were previously dependent on biochemical and morphological characteristics, including reproductive spores and colonial morphologies (Samson *et al.*, 1988), isoenzymes (Poprawski *et al.*, 1988; St. Leger *et al.*, 1992), hydrolytic enzymes (Mugnal *et al.*, 1989), and immunological cross-reactions (Shimizu and Aizawa, 1988; Tan and Ekramoddoullah, 1991). These methods are seen to be lengthy and lack potential sensitivity and rapidity to allow identification within short periods (Gasnov *et al.*, 2005). Precise EPF detection systems of high specificity and sensitivity levels could however benefit from advances in biotechnology presenting high levels of accuracy and reproducibility (Enkerli and Widmer, 2010).

Newly developed techniques based on quantitative PCR (qPCR) and loop-mediated isothermal amplification (LAMP) assays are highly reliable for microbial quantification and identification, respectively. LAMP is the molecular diagnostic technique which was first developed and documented for its ability to rapidly amplify nucleic acids under

isothermal conditions using DNA polymerase enzymes through the activity of strand displacement (Notomi *et al.*, 2000). Sensitivity, specificity, and rapidity of LAMP reactions were confirmed in different studies (Thekiso *et al.*, 2005, 2007; Lau *et al.*, 2010; Karthik *et al.*, 2016), but rarely explored for identification of environmental fungal samples including EPFs. Molecular detection of *B. bassiana* from pure cultures, or environmental samples obtained from soils or insect cadavers before isolation is crucial (Enkerli and Widmer, 2010). Therefore, recent studies were conducted with relevance to molecular techniques developed to do identification of EPF strains in the environment (Biswas *et al.*, 2013). The aim of this chapter was to develop species-specific primers based on LAMP assays for detection of *B. bassiana* from pure cultures and environmental samples.

6.2 Materials and methods

6.2.1 Soil sample collection

Soil sampling procedure was based on the methods by Goettel and Inglis (1997) and Meyling (2007). Soil samples were collected from different localities under dryland wheat production in SA. Samples were obtained from 5 SA provinces (Free State, Kwa-Zulu Natal, Mpumalanga, Western Cape and Northern Cape). Soil samples were randomly obtained (using an auger) from a plough depth of 10-15 cm of three different sampling units per area, mainly from the A horizon. Soil samples representing one sampling area were mixed together (pooled) and transported to the Agricultural Research Council (ARC) – Small Grain in zipper block plastic bag held in bigger

containers for storage. On arrival at ARC-Small Grain, samples were stored at room temperature and processed accordingly within 48 hours.

6.2.2 Fungal isolation

6.2.2.1 Soil suspension and selective medium

A soil suspension was prepared from each pooled sample (based on locality) by adding 1 g of soil to 9 ml of sterile distilled water (Dong *et al.*, 2016) amended with 0.01% Break-Thru® in 50 ml centrifuge tubes (Fig. 6.1a). To obtain a homogenous stock solution, the soil suspension was shaken for 15 minutes using a vortex. A 1ml stock suspension from each sample was transferred into 2 ml tubes (Eppendorf, SA). A 10 – fold dilution series was prepared by adjusting the dilution of 100 µl suspension to 1000 µl (1ml) to obtain concentrations of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} per sample. Aliquots of 50µl from each of 10^{-2} , 10^{-3} and 10^{-4} concentrations were inoculated on *B. bassiana* selective medium (65mm plate) (Fig. 6.1b) based with Sabouraud dextrose agar (SDA containing dodine (0.55 g/L⁻¹) and (0.005 g/L⁻¹ chlortetracycline (Sigma - Aldrich, now Merck (Pty) Ltd, Germany) (Chase *et al.*, 1986). Plates were incubated at 25 ± 2 °C for 7-14 days.

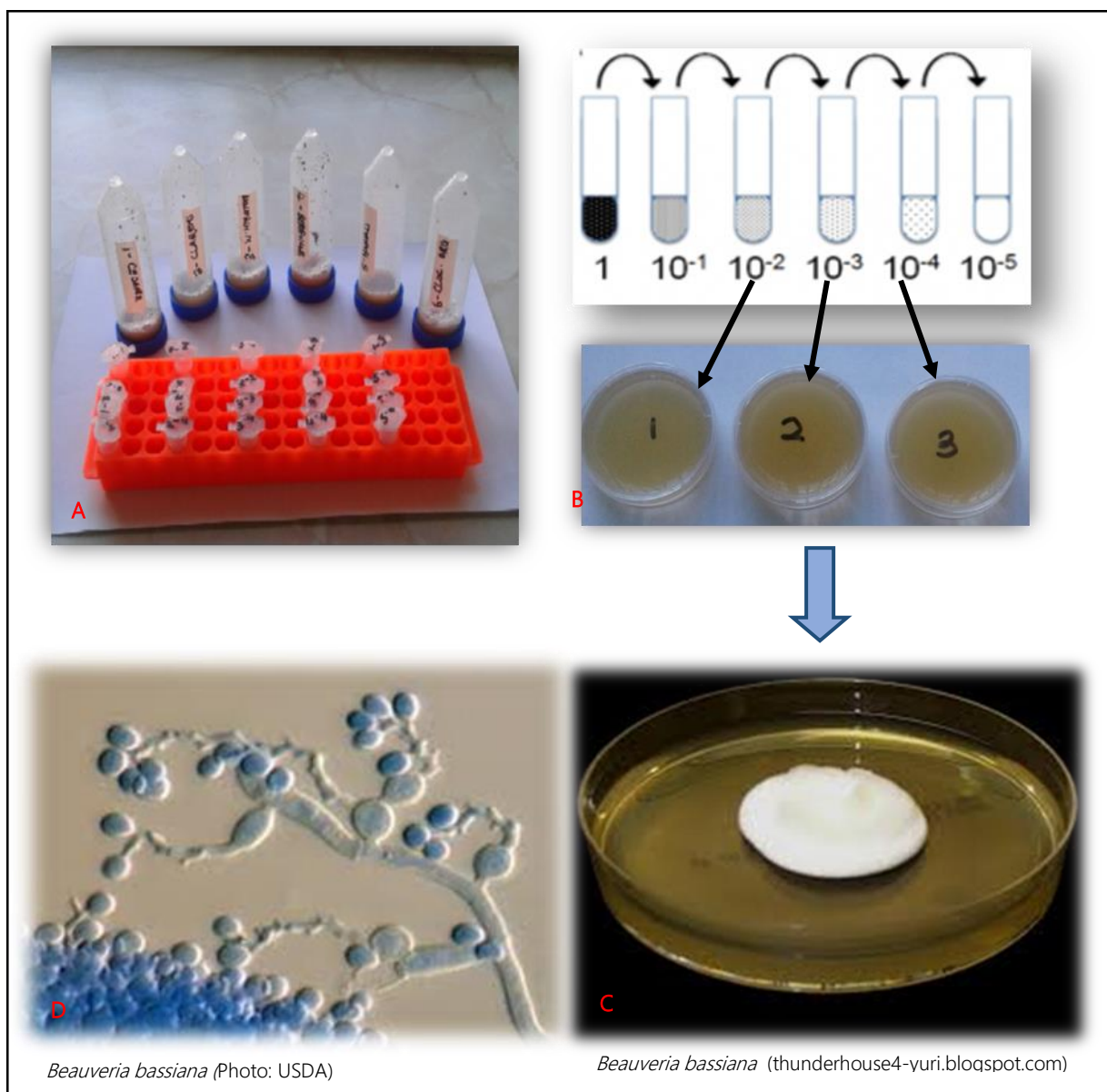


Figure 6.1: Fungal isolation from soil stock solutions derived from different localities' soil samples. **(A)** Soil suspensions diluted in 10- fold series from respective stock solutions; and **(B)** aliquots from three different concentrations (10^{-2} , 10^{-3} & 10^{-4}) were plated on selective medium for specific isolation of *B. bassiana*. **(C)** Selective growth of *B. bassiana* isolated following inoculation of 50 μ l – aliquots from the soil suspension. **(D)** Microgram showing *B. bassiana* conidiospores growing from the hyphae on the growth medium.

6.2.2.2 Insect - "Galleria" bait method

Frequent recovery of hypocrealean EPFs using selective medium tends to allow selection of saprophytic fungal complex than infective types. Alternatively, the use of insect bait method was seen to be a standard protocol for isolation of fungi from the

soil samples (Zimmermann, 1986). A highly susceptible bait insect, the great wax moth larvae, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) were trapped in moist soil samples contained in bottles with perforated lids as described by Meyling (2007) (Fig. 6.2a).

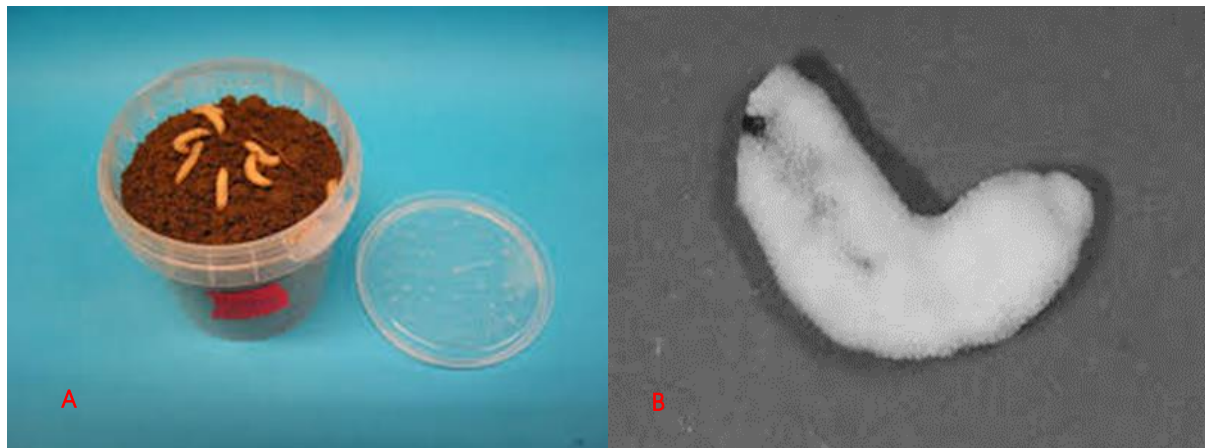


Figure 6.2: *Galleria* baiting method as described by Meyling (2007). **(A)** Larvae trapped in soil samples maintained in the containers covered with perforated lids (to allow aeration) and incubated at 25 ± 2 °C for 10-14 days. **(B)** Infected cadaver showing *B. bassiana* fungal outgrowth following incubation.

Ten larvae were trapped in each of a 500 g soil sample (labelled with their respective collection sites) (Fig. 6.2a) and incubated for fungal infection at 25 ± 2 °C for 10-14 days in full darkness. Soil containers were inverted upside down to enhance contact between the larvae and the soil (Abaajeh, 2014). Samples were inspected for fungal growth at 7 days interval. Dead larvae (cadavers) were surface sterilised with 70% ethanol, blot dried with a sterile paper towel, and were later plated on moist No.1 Whatman® filter papers (Merck, SA) prior to incubation (Abaajeh, 2014), as above. Sporulating cadavers (Fig. 6.2b) were plated on *B. bassiana* selective medium (Chase *et al.*, 1986; also see section 6.2.2.1).

So far, only few fungal isolates from Kwa-Zulu Natal and the Free State were sent for accessioning at the SA National Collection of Fungi (SANCF), ARC - Plant Protection Research Institute (PPRI = currently PPR) (as shown on Table 6.1, on the map, Fig. 6.3 and Appendix 1).

Table 6.1: Strains of *Beauveria bassiana* used for investigation in this study.

<i>Beauveria bassiana</i> strains	Host/substrate origin	Locality/area	Collection date
PPRI 7598	Insect	ARC -SG fungal collection	*N/A
PPRI 7861	Insect	ARC -SG fungal collection	N/A
PPRI 23345 (SGI115)	Wheat, <i>T. aestivum</i>	Bergville, KZN, SA	19/10/2016
PPRI 23346 (SGI116)	Soil (trapping with <i>G. mellonella</i>)	Clocolan, Free State, SA	08/12/2016
PPRI 23347 (SGI117)	Soil (trapping with <i>G. mellonella</i>)	Clocolan, Free State, SA	08/12/2016
PPRI23348 (SGI118)	Soil (trapping with <i>G. mellonella</i>)	Clarens, Free State, SA	03/11/2016
PPRI 23349 (SGI119)	Soil (trapping with <i>G. mellonella</i>)	Arlington, Free State, SA	15/11/2016
PPRI 23350(SGI120)	Soil (trapping with <i>G. mellonella</i>)	Qwaqwa, Free State, SA	06/12/2016
PPRI 23351 (SGI121)	Soil (trapping with <i>G. mellonella</i>)	Harrismith, Free State, SA	07/12/2016
PPRI 23353 (SGI122)	Weevil, <i>Listronotus setosipennis</i>	Cedara, KZN, midlands, SA	15/12/2016
PPRI 23352 (SGI814)	Green stinkbug, <i>Nezara viridula</i>	Bethlehem, Free State, SA	19/02/2015

*N/A = date not known.

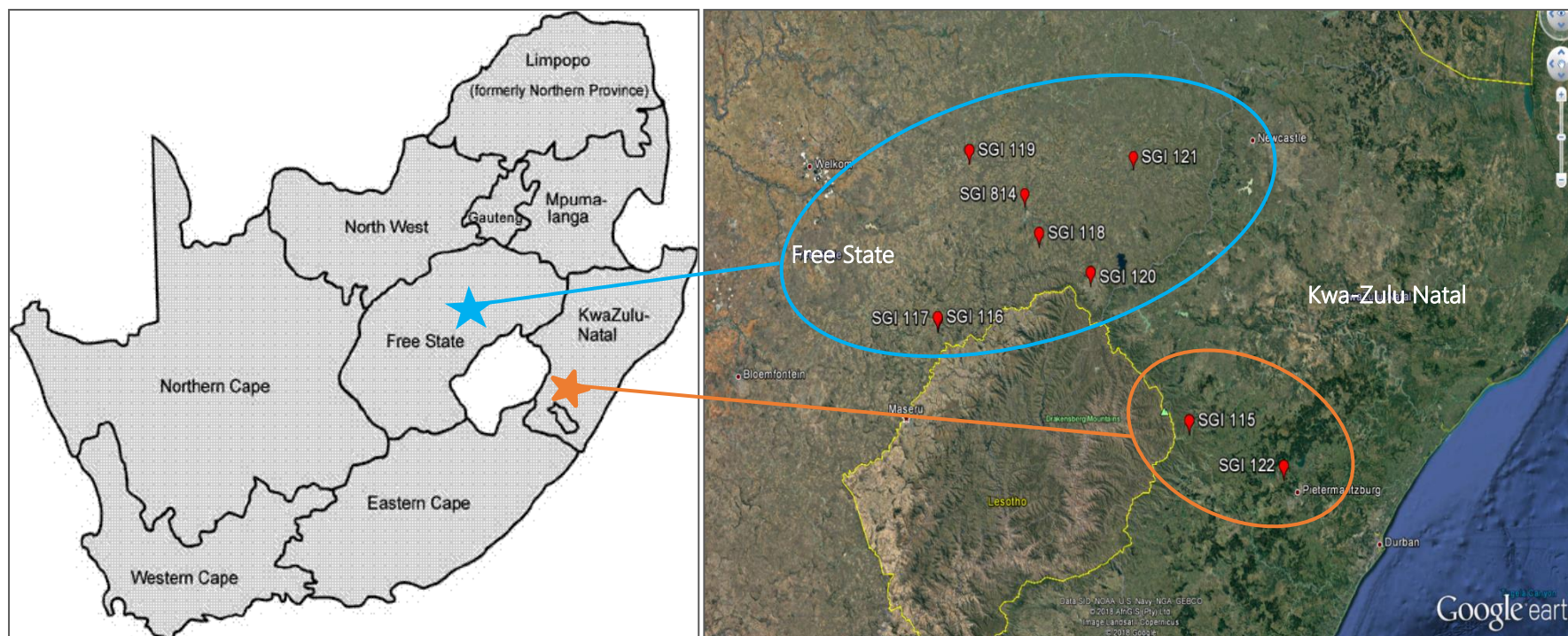


Figure 6.3: Representation of fungal isolates from different soil collection sites in dryland wheat production areas of Free State (Blue colour) and Kwa-Zulu Natal (Orange colour) provinces of SA. Insertions: the blue ring encloses localities in the Free State from which seven strains originated; while the orange ring encloses Kwa-Zulu Natal localities from which the two strains originated. Coordinates were plotted on the map by D. Human, ARC-Small Grain. Source: Google earth Map: <https://earth.google.com/web>.

6.2.3 DNA extraction and amplification

Fungal conidia from the newly isolated strains from different locality soils were inoculated on the sabouraud dextrose agar (SDA) amended with 1% yeast extract. Plates (65 mm) were incubated at 22 ± 3 °C for 14 days. Conidia were harvested with sterile camel hair brush, 0.03 g was suspended in 10ml of sterile distilled water with 0.1% Break-Thru® and the concentration was adjusted to 10^8 (equivalent of 400 propagules ml⁻¹) using an improved Neubauer haemocytometer (Marienfeld, Germany). From the stock solution, 1 ml was transferred into 2 ml eppendorf tubes. Subsequent to thorough mixing with the vortex, genomic DNA of the fungal strains was extracted following the manufacturer's instructions using fungal/bacterial DNA extraction kit (Zymo Research, SA). Genomic DNA was resuspended with an elution buffer from the kit and was stored at -20 °C. DNA concentration was quantified using the NanoDrop™ 2000/2000c Spectrophotometer (ThermoFisher Scientific™, USA), prior to PCR reactions for DNA amplification.

6.2.4 Designing of LAMP primers

To develop LAMP diagnostic assays for detection of *B. bassiana*, primers were designed targeting *B. bassiana* conidiation gene, A43 [GenBank Accession no.: EF122839.1] (Appendix 2) using Primer Explorer V4 online software (www.eiken.co.jp). The LAMP primer set with FIP, BIP, F3, B3, LF and LB primers is shown in Table 6.2. The sequences were sent for synthesis at Inqaba Biotechnical Industry (Pty) Ltd, SA. Primer length was determined by compatibility of melting temperatures of the forward and reverse primers.

Table 6.2: LAMP Primer sequences for detection of *Beauveria bassiana* isolates.

Primer	Primer sequence (5' – 3')	Primer length
FIP	GTCATATCCGAGACAGGCTGCGATGCCGAATACTGAGGTG	40 bases
BIP	TCTGGTGCCAGCGAATTAGAAGTCTCTGCTTCAATAACTGTCC	43 bases
F3	CGACAACATACTCCAGTGG	19 bases
B3	AAACTCTGGGCAAAGCAG	18 bases
LoopF	TGCAGACCGTGAGAACTTC	19 bases
LoopB	GGACATCATGTCTCGCCTC	19 bases

6.2.5 LAMP and PCR reactions

LAMP was conducted using the optimised Isothermal Master Mix kit following manufacturer's instructions. LAMP reaction mixture contained a proprietary novel DNA polymerase, Proprietary thermostable inorganic pyrophosphatase, optimised reaction buffer, MgSo₄, dNTPs, and a ds – DNA binding dye (FAM detection channel). LAMP reaction mixture included 15 µl of Isothermal master mix, 5µl primer mix, 2 µl of template DNA and 3 µl of nuclease-free water. LAMP reactions were conducted with amplification of DNA at 65°C for 30 minutes and optimum annealing temperature of 85°C - 80°C – ramping at 0.05°C second⁻¹.

Amplification of the fungal strains' genomic DNA was performed with PCR using *B. bassiana* species-specific LAMP outer primers, F3 and B3 designed in this study. PCR was conducted for the final volume of 25 µL using OneTaq® Quick - Load® 2X Master Mix with standard buffer (BioLabs, USA) which contains 20mM Tris-HCl (pH 8.9@25°C); 1.8 mM MgCl₂; 22 mM NH₄Cl; 22 mM KCl; 0.2 mM dNTPs; 5% glycerol; 0.06% IGEPAL®CA-630; 0.05% Tween® 20; Xylene Cyanol FF; Tartrazine; and 25 units/ml OneTaq DNA Polymerase. PCR reaction mixture

included 12.5 µl of 2X master mix, 1 µl of forward primer, 1 µl of reverse primer, 2 µl of template DNA and 8.5 µl of nuclease-free water. Thermocycling conditions comprised an initial denaturation of 30 seconds at 94 °C followed by denaturation at 94°C for 30 seconds for 35 cycles. The optimum annealing temperature was 57°C for 1 minute followed by 68°C final extension for 5 minutes and followed by a 4 °C hold. The amplified DNA fragments were visualized on 1% agarose gel.

6.2.5 Primer optimisation, specificity and sensitivity

Optimisation. Three sets of LAMP primers were developed specifically for detection of *Beauveria bassiana*. The primers were optimised for both LAMP and PCR reactions. Primers were optimised following six temperature regimes of 51, 54, 57, 60, 63 and 66°C.

Specificity. *B. bassiana* fungal strain cultures grown on SDAY containing 1% yeast extract were incubated at 22 ±3°C and later used to test specificity of LAMP primers (F3 and B3) following DNA extraction using the fungal DNA extraction kit as mentioned above. PCR specificity reactions were performed using the optimised annealing temperature (57°C) specific for *B. bassiana* species in this study. Specificity was tested following the same PCR conditions and DNA amplification quantified as mentioned above.

Sensitivity. Primer sensitivity was tested at different *B. bassiana* concentration levels. PCR assays which deployed F3 and B3 primers were conducted with the 10 – fold serially diluted

DNA (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5}) of *B. bassiana* PPRI 7598 stock using same PCR assay conditions mention in section 6.2.5 above.

6.3 Results

6.3.1 Fungal isolation methodology evaluation

The two isolation methods were used for the purpose of this study (no data shown), however the two methods equally isolated fungal strains. The fungal growth was not quantified since this procedure was only done for isolation and production of conidia for identification purposes using *B. bassiana* species-specific primers designed in this study.

6.3.2 Optimization of LAMP assay (FIP, BIP, F3 and B3 primers)

There were no successful reactions for LAMP assays, i.e. no development of reaction curves on real-time LAMP device and no bands were formed on gel electrophoresis (Data not shown). As a result, LAMP assay development was discontinued.

6.3.3 Optimization of F3 and B3 primers

When primers were optimized at six temperature regimes, 51, 54, 57, 60, 63 and 66°C the *B. bassiana* PPRI 7598 DNA was amplified at most temperatures of 51, 54, 57, 60, 63°C (Fig. 6.4). PPRI 7861 amplified only at temperatures 51 and 54°C and no amplification of negative controls at all temperature regimes.

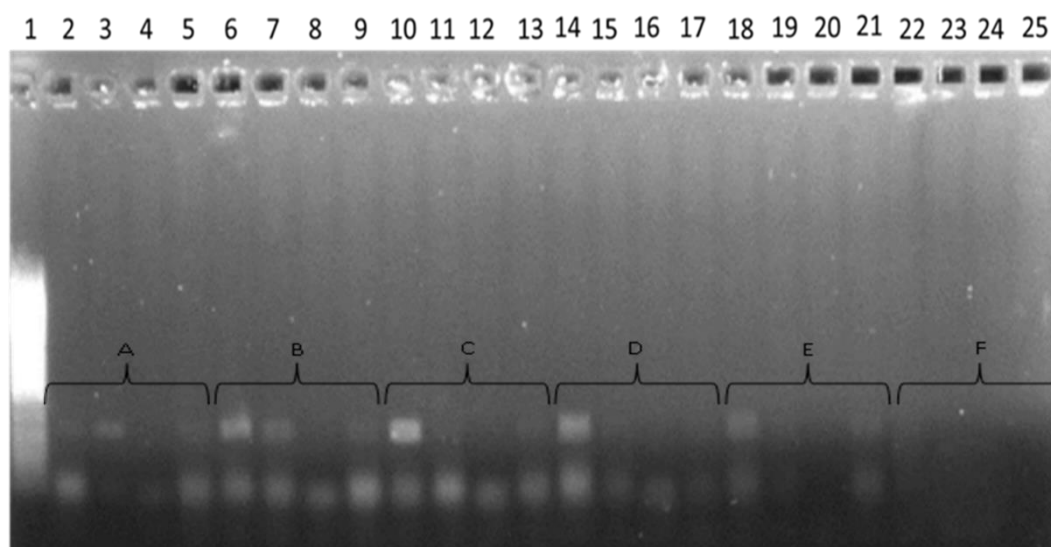


Figure 6.4: Optimisation of primers: Gel electrophoresis was run with the 100bp DNA ladder. Strains' fragments were observed at 300bp. Lane1= DNA Marker. Lane 2 = PPRI 7598; Lane 3 = PPRI 7861; Lane 4 = *Fusarium* spp. and Lane 5 = *DDW at temperature A (51 °C). Lanes 6 – 9 at temperature B (54 °C); Lanes 10 -13 at C (57 °C); Lanes 14-17 at D (60 °C); Lanes 18-21 at E (63 °C) and Lanes 22 – 25 at F (51 °C). Samples were arranged in the similar pattern across all temperature regimes. * Double distilled water.

6.3.4 PCR specificity for amplification of various South African *B. bassiana* strains

The *B. bassiana* PCR assay with F3 and B3 was evaluated for specificity in DNA detection of South African *B. bassiana* strains isolated from the soil in this study. The PCR assay amplified *B. bassiana* maintained in ARC-SG fungal collection (PPRI 7598 and PPRI 7861) newly isolated strains in this study with exception of PPRI 23345 and PPRI 23351 strains. No detection of negative controls (*Fusarium* spp. and double distilled water (DDW)) was observed. The PCR product amplification size was 300 bp (Fig. 6.5).

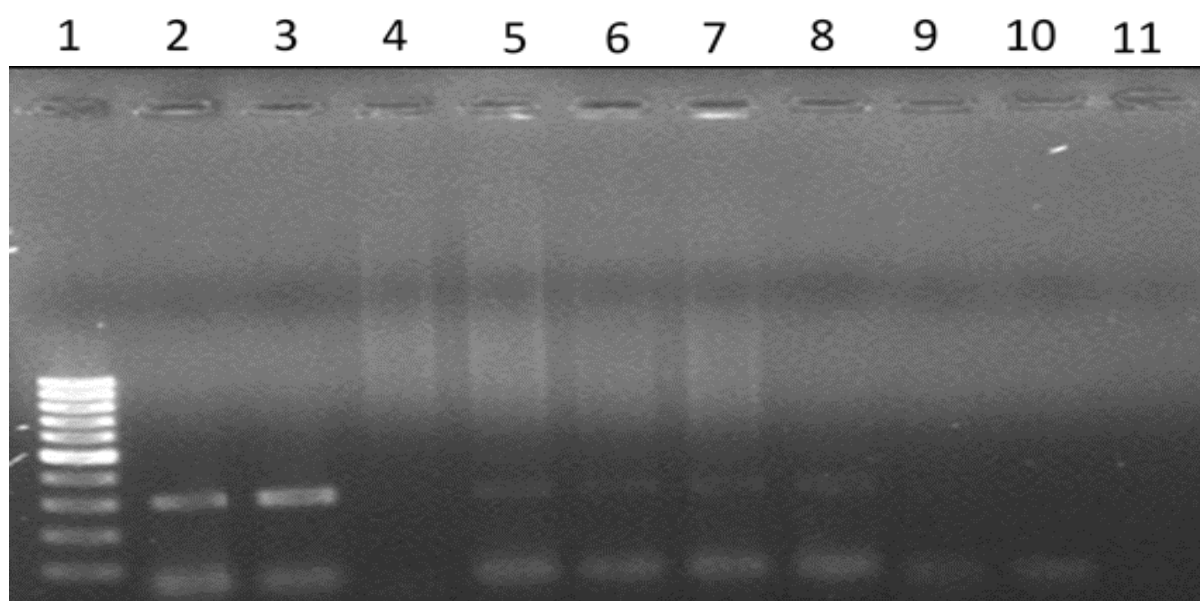


Figure 6.5: PCR specificity: Gel electrophoretic analyses of PCR products derived from the *B. bassiana* specific primers (F3 & B3) amplification as separated in 1% agarose. Gel electrophoresis was run with the 100bp DNA ladder. *B. bassiana* strains' fragments were observed at 300bp. Lane1= DNA Marker. Lane 2= PPRI 7861; Lane 3= PPRI 7598; Lane 4=PPRI 23345; Lane 5= PPRI 23346; Lane 6= PPRI 23347; Lane 7= PPRI 23348; Lane 8 = PPRI 23350; Lane 9 = PPRI 23351; Lane 10 = *Fusarium* spp and Lane 11 = DDW.

6.3.5 Primer sensitivity

Only *B. bassiana* strain (PPRI 7598) aliquots were detected and amplified, while negative controls (*Fusarium* and DDW) could not be detected by the tested primers. However, amplification varied between the three concentrations (Fig. 6.6). There was a high level of primer sensitivity at the highest concentration, 10^{-1} than at two successive concentrations (10^{-2} and 10^{-3}).

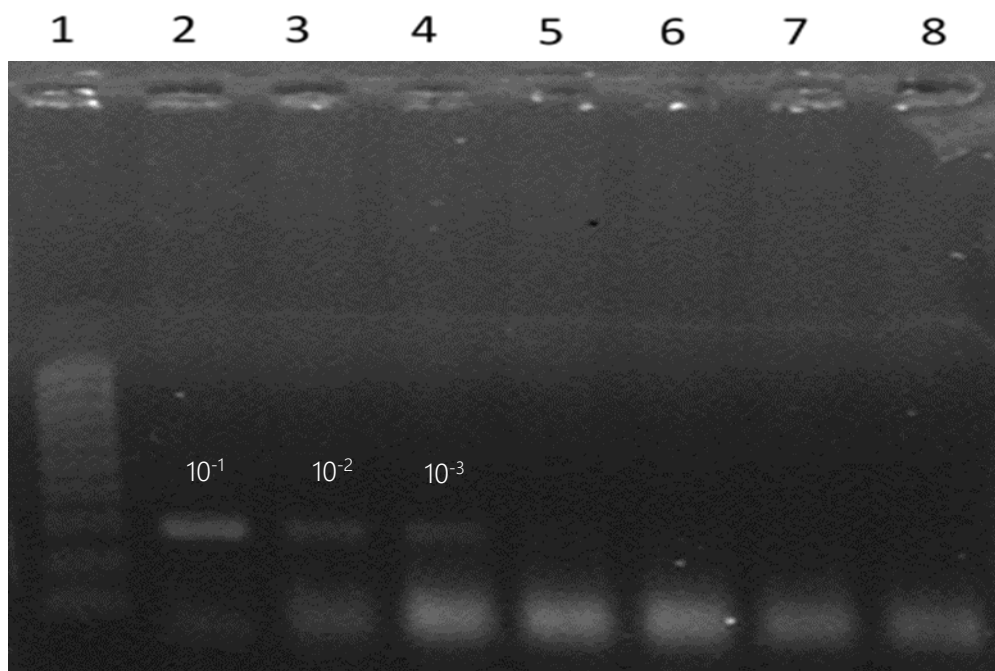


Figure 6.6: Primer sensitivity: PCR product was visualized in 1% agarose with ethidium bromide staining. Gel electrophoresis was run with the 100bp DNA ladder. *B. bassiana* strains' fragments were observed at 300bp. Lane1= DNA Marker; lanes 2, 3 & 4 = PPRI 7598 aliquots at 10^{-1} , 10^{-2} and 10^{-3} concentrations; lanes 5, 6 & 7 = *Fusarium* aliquots at 10^{-1} , 10^{-2} and 10^{-3} concentrations and lane 8 = DDW (negative control).

6.4 Discussion

The aim of this study was to isolate unknown *B. bassiana* strains from the wheat fields in SA and develop species-specific molecular assays for detection of *B. bassiana* isolates recovered from South African soils. The EPFs naturally inhabit soil (Hajek, 1997) and their relative abundance in different soils has been reported in several studies (Bidochka *et al.*, 1998; Keller *et al.*, 2003). As such, many species belonging to the Phylum Ascomycota notably *Beauveria* spp., *Metarhizium anisopliae* and *Paecilomyces* spp. inhabit soils for some part of their life cycle living outside the insect host (Keller and Zimmermann, 1989). Laboratory measures for isolation of soil-borne EPFs such as *Beauveria bassiana* and *Metarhizium anisopliae* have been developed and are widely applied (Meyling, 2007). Most popular

methods are selective medium soil (suspension) plating and *Galleria* baiting. Interestingly, the *Galleria* baiting method has been the simplest and efficient method reported from the previous studies (Meyling, 2007; Tuininga *et al.*, 2009). In this study, both methods were employed to select the best method which could isolate mainly *B. bassiana* isolates from South African soils as the source of DNA material. Indigenous fungal genomic DNA was required for optimisation of the newly developed PCR assay for *B. bassiana* for detection of South African *B. bassiana* isolates.

In this study, 7 *B. bassiana* strains were isolated from soil samples collected from wheat fields, whereas one was isolated from *Listronotus setosipennis* (Coleoptera: Curculionidae). Strains were isolated using both soil suspension and selective medium and the *Galleria* - bait method, although the *Galleria* - bait method was more efficient than soil suspension and the selective medium. The choice of the isolation method in this study was entirely influenced by simplicity and effectiveness of the method based on our observations and literature. When the two methods were applied, our findings confirmed the effectiveness of the *Galleria* baiting method over the traditional selective medium plating method for many reasons. (a) The disadvantage with soil suspension plated on selective medium is the possible risk of overgrowth of opportunistic fungi on the media, or (b) the risk of the ultimate failure to select the target fungi from 1 g of the soil sample since EPF maintain a clumped distribution in soil (Meyling, 2007). (c) Additionally, for the convenience of this study, the basic need was to get a cost-effective isolation method, whereas the selective media require some expensive additives (mostly imported) to prevent opportunistic fungal overgrowth (Meyling, 2007).

Based on our observations, the *Galleria* baiting method underscored positive selective isolation and sensitive detection of *B. bassiana* fungus from the soil samples. However, each of the two methods portrayed some advantages and disadvantages as highlighted by Meyling (2007) (Table 6.3).

Table 6.3: Observations confirming advantages and disadvantages of using different isolation methods as highlighted by Meyling (2007).

Isolation method	Advantages	Disadvantages
Soil suspension and selective medium	Obvious possibilities to quantify inoculum coliform forming units (cfu) or colonies	High risk with overgrowth of opportunistic soil fungi on media was evident
	Quantitative data possibly generated (data not analysed)	Small soil sample (1 g) used creates low chances of selecting fungus from smaller soil samples
	Parametric data accessible to allow the use of standard statistical analyses (ANOVA) (data not analysed)	Low fungal dilution values since samples are diluted from 1g soil sample
Insect bait method	Use of <i>G. mellonella</i> became a very sensitive detection method	Some insect species may select for specific fungal pathogens
	EPFs are selectively isolated, <i>Beauveria</i> spp. most observed	Moist soil may enhance the infection of nematodes and not fungi
		Difficult to quantify inoculum levels

An attempt by this study to develop LAMP assay for detection of South African *B. bassiana* isolates was unsuccessful because LAMP reaction did not occur at all despite repeated

troubleshooting at different temperatures. It is suspected that the 6 primers (FIP, BIP, F3, B3, LF and LB) are not melting at one single temperature as expected hence no reaction was taking place. Under normal circumstances the optimal reaction temperature of LAMP ranges between 60°C and 65°C (Notomi *et al.*, 2000), however depending on the melting temperatures of the primers that optimal reaction temperature can sometimes be outside that range.

This study has successfully developed conventional PCR using F3 and B3 primers targeting the A43 gene of *B. bassiana*. The optimal amplification temperatures for the new PCR assay are 54°C and 57°C as judged with the band intensity on the agarose gel electrophoresis.

When tested for sensitivity, the new PCR assay detection limit was down to 10^{-3} of serially diluted *B. bassiana* PPRI7598 DNA. This is equivalent to 0.4 spores ml⁻¹. This is an indicator that the A43 gene-based PCR developed in this study will be sensitive enough to detect the *B. bassiana* fungi even when it is in low concentrations in the soil, plant or in arthropods. This detection limit is similar to the *ef1α* gene-based PCR assay developed by McKinnon *et al.* (2018) which also displayed detection sensitivity of 0.32 pg/μL for *Beauveria* spp. Specificity results in the current study confirmed that the new A43 gene PCR accurately amplified *B. bassiana* species. No amplification occurred from the negative controls which were *Fusarium* spp and DDW (no template DNA).

This is a good indicator that the new assay is species specific for *B. bassiana* whereby it does not amplify other fungi as a no template DNA (negative) controls. The assay could amplify the PPRI 7598 and PPRI 7861 strain which are both maintained in the ARC-Small Grain Culture

Collection. The PCR assay also amplified 6/8 (75%) strains isolated from the soil in this study. This shows the capability of the assay to detect various *B. bassiana* strains/isolates originating from South Africa. However, we could not amplify South African *B. bassiana* using SCA PCR published by Castrillo *et al.*, (2003). Furthermore, Biswas *et al.* (2013) also reported that the same strain-specific primers failed to amplify a different *B. bassiana* strain. As a result, it has been important for us to develop PCR assay that could amplify South African *B. bassiana* strains. Our assay has further indicated that the identity of two other strains, PPRI 23345 and 23350 will have to be reconfirmed by other methods since they could not be detected by our *B. bassiana* specific primers. Generally, the findings from this study are also in agreement with McKinnon *et al.* (2018) to infer that the *B. bassiana* gene- based primers demonstrated high levels of sensitivity and specificity for monitoring *B. bassiana* in in non-sterile soil.

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CHAPTER 7: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

7.1 Introduction and objectives of the study

In SA, wheat is the second most grown cereal crop which is predominantly grown under dryland and irrigation systems. Dryland production comes from both summer and winter rainfall regions. However facing the adverse climate change featuring drought and negative effects brought by pests and disease, wheat production currently encountered 16% decline between the 2007/2008 and 2016/2017 seasons (SAGL, 2018). Erratic rainfalls that occur in the Free State region make it difficult to control common pests of wheat particularly the RWA using chemical pesticides, under these dry conditions (Du Toit, 1992) .

The RWA is a globally known economic pests among cereal crops. Hence its sap sucking mode of feeding inflicts damage to the host crop leaves, negatively reducing wheat yields, the use of the host plant resistance strategy was seen environmentally and economically reliable in sustainable agricultural systems (Tolmay, 2006; Porter *et al.*, 2009). However, development of host plant resistance breaking biotypes continue to pose a serious threat to wheat producers in the SA (Jankielsohn, 2017). Alternatively, the use of EPFs as efficient biological control agents in conservation biological control strategies is globally adopted (Meyling, 2007). Following different initiatives such as integration of the EPF with the host plant resistance to improve efficacy of EPF *B. bassiana* against pests in SA (Hatting *et al.*, 2004), EPF *B. bassiana* is incorporated into the IPM strategy as mycopesticide products (Hatting *et al.*, 2018) and/or endophytes, the attribute which was confirmed in the recent review

(Vega, 2018) for pest and disease control. The sole role of pest management that is ascribed to the use of fungal endophytes has contributed to initiation of the current study.

Major findings in this study support the hypothesis that “the EPF, *B. bassiana*, can be artificially induced as an endophyte in selected wheat cultivars to enhance the development of systemic resistance against the *D. noxia*”. Therefore, the aim of the current study was to establish *B. bassiana* as an endophyte in wheat and evaluate its endophytic effect against *D. noxia*.

The objectives of the study were to:

- conduct laboratory bioassays to select *B. bassiana* isolates highly virulent to Russian wheat aphid, *D. noxia*.
- evaluate different inoculation techniques for establishing *B. bassiana* as endophyte in *T. aestivum* and measure its impact on seedling growth.
- evaluate the effect of endophytic *B. bassiana* on the Russian wheat aphid through the measurement of intrinsic rate of increase and other parameters.
- develop species-specific LAMP assays for detection of *Beauveria bassiana*.

However, general pointers of the study are briefly discussed and conclusions be drawn based on the findings from each objective of the study presented as individual chapters (Chapters 3, 4, 5 & 6). Lastly, recommendations for future studies emphasise the need to: **a)** address observed limitations of the study; and **b)** improve the potential of the candidate endophytic fungal strain’s virulence and extended persistence for sustainable use by SA farmers.

7.2 General discussion

The current study proposed the development of a candidate strain of the EPF *B. bassiana* suitable for endophytic establishment in *T. aestivum* cultivars. The EPF *B. bassiana* strain PPRI 7598 was virulent to RWA at relatively higher concentration of 1×10^8 conidia⁻¹ compared to its counterpart commercial PPRI 7861. In agreement with reported results by Sakupwanya (2007), our results confirmed that percentage mortality of aphids occurs over time with increasing conidial concentrations and that entomopathogens are however slow acting although environmentally safe as compared to the chemical pesticides. The passaging of an EPF through an insect host is advantageous in that it enhances or reactivates the strain virulence (Song and Feng, 2011), although this exercise did not improve virulence of the plant derived isolate. Therefore, it is highly imperative to use a fungal strains derived from the target pest (e.g PPRI 7598IN), to effectively control its host pest. For the same notion, Lockwood (1993) discourages the use of imported isolates since the EPFs efficacy is environmentally dependent. Importantly, knowledge of the strains' background provides the basis for their use against specific pests (Quesada-Moraga *et al.*, 2007).

Although a degree of impediment for control of aphids was observed with the endophytic *B. bassiana* isolate PPRI 7598PL, Wagner and Lewis (2000) also reported results with no significant difference between the original isolate and the one derived from the corn (Poaceae) plant against *Ostrinia nubilalis*.

Successful endophytic establishment of *B. bassiana* in potted wheat seedlings was evident for at least 14 days after inoculation using seed treatment. This method is however, simple and

cost effective hence it is the most adapted following leaf spraying in most studies (McKinnon *et al.*, 2017). However, persistence of *B. bassiana* candidate strain PPRI 7598 was not assessed beyond 14 days of inoculation, although it was noted in a similar study by Guesmi-Jouini and Garrido-Jurado (2014) that establishment based on fungal recovery from leaves, stems and roots declined over time. Results in this study confirmed that when *B. bassiana* was inoculated in wheat, colonisation became systemic in plants. As such, the fungal endophyte was recovered from different plant parts confirming the systemic transmission, although molecular verification was not successful. Interestingly, fungal colonisation of plants did not induce physical damage or result in disease symptoms to plants, instead, colonisation resulted in improved plant growth in wheat seedlings. In agreement with other studies, positive plant growth following *B. bassiana* colonisation was also confirmed (Akello *et al.*, 2008; Dara, 2013; Jaber and Enkerli, 2016; Sánchez-Rodríguez *et al.*, 2018). However, decline in the endophytic strain persistence in plants over time which was observed in this study, also reported by Guesmi-Jouini and Garrido-Jurado (2014) could implicate the strain quality. Besides, this could be an effect of environmental factors surrounding plants.

Furthermore, the endophytic potential of the candidate strain for control of aphid demographic growth was confirmed in this study. However, the strains affected different population growth parameter differently, significantly ($P < 0.05$) reducing the RWASA1 net reproductive rate in the endophyte treated plants. Population growth rates varied significantly ($P < 0.05$) between the two treatment levels, the endophyte inoculated and control plants. Similar trend was also observed across the cultivars, on the two RWASA1 susceptible cultivars (Molopo and Kariaga) as compared to Gariep (the resistant cultivar). Remarkably, assessment of the feeding intensity of the RWA on endophytically inoculated cultivars which contributes

novelty in this study, was performed on both treatment levels using the damage rating index adapted from the ARC- Small Grain (Tolmay, 1995). The resistance seen against the RWA herbivory in Gariep could ascertain an integration of the endophyte and host plant resistance potential to mitigate resistance breaking trait in *D. noxia* biotype (RWASA1).

7.3 Concluding remarks and recommendations

Chapter 3:

When the candidate *Beauveria bassiana* strain PPRI 7598 was assayed against the *D. noxia*, the findings of this study confirmed the potential of this isolates for control of *D. noxia*. However different factors might have influenced the nature (lower mortality) of the results obtained in this study. The strain quality could also be a contributing factor to low efficacy of the strain and could ultimately affect the persistence of the inoculum and its shelf life.

Recommendations for future research:

- Future studies are required to manipulate PPRI 7589 strain, particularly the plant derived isolate (PPRI 7598PL) to improve its conidiation (maybe through repeated passaging measures), virulence stability and persistence and resistance to biotic and abiotic stressors.
- Additional studies to explore insect host defense mechanism against fungal infection are also essential.

- Subsequently, such advances could be essential for development of efficient commercial candidate strains with extended shelf life (Mascarin *et al.*, 2016). It highly imperative to develop a strain with sustainable virulence under field conditions.
- It is important to pioneer studies that can provide prerequisite knowledge required before formulation and registration of a commercial product.

Chapter 4:

The findings of this study suggests the potential use of PPRI 7598 as an endophyte in the agroecosystems for improvement of wheat growth, possibly even in other agricultural commodities. The following conclusions are drawn from the findings:

- The use of seed treatment method was found to be the most convenient and cost-effective method for farmers, and most adapted in most endophytic related studies.
- The uptake and systemic spread of endophytic *B. bassiana* via seed treatment (Behie *et al.*, 2015) holds significant potential as an economical and practical inoculation method; particularly in SA, where wheat seeding rates range from 15 to 30 kg per hectare (ARC-Small Grain, 2018).
- Through this study, endophytic *B. bassiana* PPRI 7598IN presented a positive potential for plant wheat seedling growth and improved wheat yield.

Recommendations for future research:

- Based on the findings of this study, future research should be considered to explore the potential vertical transmission of *B. bassiana* to wheat grain as reported in opium poppy seeds (Quesada-Moraga *et al.*, 2014). Establishment of vertically-transmitted endophytes which can be transferred directly from the host plants (parents) to their progenies (Saikkonen *et al.*, 2002) is one of the studies targeted for control of pest in most agronomic crops in SA. This strategy could be a relief towards intensive mass production and application of the fungal conidia under field conditions.
- Another study to evaluate and improve persistence of the endophyte into the next generation. Improved strain persistence could conveniently work to farmer's advantage, particularly resource-poor or emerging farmers in SA by reducing pesticide costs.

Chapter 5:

On the question of whether an endophytic *B. bassiana* strain present in wheat seedlings had negative impacts on RWA, the results confirmed that *B. bassiana* strain PPRI 7598 had significantly reduced aphid reproductive rate and subsequently the population growth. Irrespective of relatively low endophytic colonisation rates of *B. bassiana* in all tested wheat cultivar seedlings, a significant effect on *D. noxia* population was evident. We therefore provide for the first time the evidence of the endophytic *B. bassiana* potential against the RWA performance.

- Generally, endophytic establishment of the EPF, *B. bassiana* in wheat cultivars holds a potential for a new and sustainable plant protection strategy against the Russian wheat aphid.
- The integration of the biological systemic pest control and cultivar resistance against insect herbivores observed in this study showed full potential for insect pest management and mitigation of resistance breaking biotypes (as seen with the cultivar Gariep).

Recommendations: Positive plant endophytic colonisation of plants attracts future research to exploit the potential of the vertical transmission notion (Quesada-Moraga *et al.*, 2014) for suppression of insect herbivory and common in SA plant diseases like *Fusarium* head blight, Karnal bunt. Furthermore, the following areas also need attention for future research:

- Development of molecular methods (e.g. qPCR) for quantification of endophytes in plants and evaluation of endophytic *B. bassiana* persistence in plants.
- Basic and applied research in developing techniques for isolation and identification of endophytes
- Incorporation of methods for assessment of agronomic impacts on endophytic colonisation

Chapter 6:

The objective of this chapter was to develop a molecular protocol specifically for detection of fungal strains recovered from SA soils. Newly developed PCR assay targeting the A43 gene

showed reasonable sensitivity and specificity levels to the candidate *B. bassiana* strain PPRI 7598 together with other involved strains in the current study. However, the development of LAMP assay was unsuccessful due to suspected inability to find single melting/annealing temperature for six primers which are included in the LAMP reaction.

Recommendations:

- The newly developed PCR assays need to be used for screening larger sample size of *B. bassiana* isolates to confirm its detection efficiency of this fungi from soil, and various plant parts, wheat in particular.
- Further attempts to develop LAMP assays with primers designed from other gene targets are recommended.

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APPENDICES

Appendix 1

Fungal cultures for accessioning in SANCF, ARC-PPRI

Collection Date	Collector	Species	Host / substrate	Area	Coordinates	SGI Number	PPRI Accession no.
19/10/2016	Bomikazi Gqola	<i>Beauveria bassiana</i>	Wheat, <i>Triticum aestivum</i>	Bergville, KZN, SA	S 28°78'94,7" E 029°33'32,4"	SGI 115	23345
08/12/2016	Astrid Jankelsohn	<i>Beauveria bassiana</i>	Soil (trapping with <i>Galleria mellonella</i>)	Clocolan, Free State, SA	S 28°84'39,7" E 027°69'64,4"	SGI 116	23346
08/12/2016	Astrid Jankelsohn	<i>Beauveria bassiana</i>	Soil (trapping with <i>Galleria mellonella</i>)	Clocolan, Free State, SA	S 28°85'12,8" E 027°70'00,0"	SGI 117	23347
03/11/2016	Thobeka Khumalo	<i>Beauveria bassiana</i>	Soil (trapping with <i>Galleria mellonella</i>)	Clarens, Free State, SA	S 28°22'37,6" E 028°25'10,3"	SGI 118	23348
15/11/2016	Lisemelo Motholo	<i>Beauveria bassiana</i>	Soil (trapping with <i>Galleria mellonella</i>)	Arlington, Free State, SA	S 27°56'63,3" E 027°53'35,4"	SGI 119	23349
06/12/2016	Lisemelo Motholo	<i>Beauveria bassiana</i>	Soil (trapping with <i>Galleria mellonella</i>)	Qwaqwa, Free State, SA	S 28°34'18,0" E 028°48'10,7"	SGI 120	23350
07/12/2016	Lisemelo Motholo	<i>Beauveria bassiana</i>	Soil (trapping with <i>Galleria mellonella</i>)	Harrismith, Free State, SA	S 27°55'90,6" E 029°04'55,4"	SGI 121	23351
15/12/2016	Lorraine Strathie	<i>Beauveria bassiana</i>	Weevil, <i>Listronotus setosipennis</i>	Cedara, KZN, midlands, SA	S 29°32'53.25" 030°16'00.03"	SGI 122	23353
19/02/2015	Justin Hatting	<i>Purpureocillium lilacinum</i>	Green stinkbug, <i>Nezara viridula</i>	Bethlehem, Free State, SA	S 28°09'53.32" E 028°18'17.11"	SGI 814	23352

Appendix 2

GenBank: EF122839 *Beauveria bassiana* conidiation gene A43 sequence

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1  gaaccaatgt  agaaaaatgt  cgagcagaag  ctcatctgcc  atcatgaccg  acaccagttg
61  aataacatgt  ctagcgatgc  aaggtcattg  tcaatgcgaa  gaggagcagc  ccttaaccat
121 agccgccgag  catcatgaaa  aaagaatgcc  acctcggaaa  gcccgtttga  aatcactctt
181 gcaccagaga  tcagcgtgca  ggtctagctg  catatggcga  gatcggactt  tggcgtcgat
241 cggagactgc  gctcacggca  gcccaacgac  atgcttcccc  aggcaatata  ccaaaaagta
301 tccttagact  acacgccttt  agcattaccc  gcgtcttgag  atatgtacat  cccctaact
361 tcgttgactt  tgctgtctc  acaacctcag  caagtctgca  gcaaggcatg  gcgccataac
421 tacattggaa  catgagtgca  tcatgcaaag  acaagaggct  ggctgaagtc  tgcatacccg
481 gagaacttgc  acatttatctg  ctgacagttg  cgtcctatat  tctgttctct  gcggtttctt
541 gcaacgctcat  ctctatggtc  cagcctctgt  ctctacgttg  cttctcaaac  ttgcatgatt
601 tagttgagaa  ttgccacgtc  attgcaagag  tcttatcttc  aacgattgaa  acatgtcctc
661 tgcttgcact  tctggtattg  caactatggg  cctgcaacaa  tgtctatcac  atcctgaagg
721 ccgaccggca  tagctcgaaa  acgcgttcag  cttgctctcc  tcagtgttcg  cttcgagaga
781 gccgagagac  tcgacaacat  actccagtgg  cgtcagaagt  agcaaataga  ttgctctaaa
841 ccgctcgcgg  atgccgaata  ctgaggtgcg  gggaagttct  cacggtctgc  agcatagtgc
901 aagcactggc  agcctgtctc  ggatatgact  ttgatatgtc  ggtgccacg  aattagaagt
961 gacgacgttt  ggacatcatg  tctcgctctg  gacagttatt  gaagcagaga  gagcacgcca
1021 tccggcgcat  catttggtac  gtactggcca  atctctcagc  tatataacgg  ctgctttgcc
1081 cagagtttct  atggccgtct  cttcgatcaa  cagcaacaca  attctacgca  aacatcaatt
1141 ctacactcat  ctgtcaaaaa  cactttcttg  acatccatca  tggcttttgc  tctccccatc
1201 cacaaccttc  ccatggacaa  gccggtcgcc  tctgaggtct  ttgagtacga  aaacgagacc
1261 ttttacaatt  ggctgtctac  ggagacggag  cgtgccaca  ttgccagcat  gctcaacgtg
1321 gaaagtaaga  gaaaccaagt  cacttgaaac  ccttcgcagg  aaggagaaga  agaagaagaa
1381 gaagaagaag  aatgcattgca  aatcatcgct  ggctaacatg  acgtttcctt  ccacagcgag
1441 cgagttgaag  ttcaggggca  acaactttct  ccaggaccgc  tccgagtgcg  gcaagtgcgg
1501 caagtattcg  ggcattggacg  atttcgtcca  caacgccctc  tatcgggca  tccactcggt
1561 cgagttcatg  aaggactttc  ttctaggaaa  gactgagcag  gccaccccg  aactgagca
1621 cgagtcggtt  tgctccccgt  gcgacaccaa  gcacgatgaa  aacaagagct  ggaaggcctg
1681 gcctccctgg  cgcaactggg  agcgtgtaca  cttttggctg  gagagcgagg  aatcctttga
1741 caagtattac  cgtgtaattt  gttgttctc  gatagtggc  ttttttttc  cgcagtgtac
1801 tcgtcgagcc  aggcactgtg  gggccagaat  agcttagagt  ggctagatgc  agtaccagcc
1861 atgacgtact  acctacctac  agaactgcc  gtcttcattc  cgtgcacact  aagcttctgc
1921 tgctgatagt  tccatatttt  taaatgtata  tatataaagt  aaataaaatt  tcattacagg
1981 cttgctatat  tgctgtata  cctggctgat  cagcagattt  agcaggctgc  aattgttatc
2041 gtcaaaaata  tccatcgta  cacgtgacgt  ttcgagctca  cactcttccc  ctttcggctt
2101 atcggcaagc  gcaagctcct  gataacgaac  ggacaagaag  ttgtctctcc  gcgaaatttc
2161 atcaccaacg  tcatccctac  agccacatac  ccaacgccac  tgcaacgctc  tctcaaccaa
2221 atttttttgc  cccgtcgttg  ccatctcaat  cagccaaaat  ggacatgtat  gccttcccac
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2521 ttcccaggga  gcaaaggcta  actgcacacc  aacccatcaa  aacaggcttt  cggcggcctc
2581 gtcgaccact  gcttcacctc  gtgcgtcgac  gacttcacct  ccaaggccct  ctcatcccg
2641 gaaaacggct  gcatcaaccg  ctgcgtctc  aagtggatgg  ctcccagca  gcgcgtcagc
2701 gaccgcttcc  aggagcacia  cgccagctg  tcgcagcagc  tgcagaacta  gggctgcacc
2761 acagctgact  cgtctttttt  ttgg
```