BIOLOGICAL CONTROL OF GASTROINTESTINAL NEMATODES OF SMALL RUMINANTS, USING *BACILLUS THURINGIENSIS* (BERLINER) AND *CLONOSTACHYS ROSEA* (SCHROERS)

By

Mahlatse Annabella Baloyi

Submitted in fulfillment

Of the requirement of the degree of

Master of Science

In the

Discipline of Plant Pathology

School of Agricultural Sciences and Agribusiness

Faculty of Science and Agriculture

University of KwaZulu-Natal

Pietermaritzburg

Republic of South Africa

MARCH 2011

ABSTRACT

Gastrointestinal nematode parasites cause great losses in the production of small ruminants through reduced productivity and the cost of preventive and curative treatments. Because of the threat of anthelmintic resistance, biological control of sheep nematodes has been identified as an alternative to anthelmintic drugs. *Bacillus thuringiensis* (*Bt*) (Berliner) and *Clonostachys rosea* (Schroers) have been widely studied as biocontrol agents. *B. thuringiensis* has been used for the biocontrol of insects and *C. rosea* has been successfully used as biocontrol agent of *Botrytis cinera* (De Bary) in plants.

B. thuringiensis and *C. rosea* strains were isolated from soil collected from the Livestock Section at Ukulinga Research Farm, University of KwaZulu Natal, Pietermaritzburg. Twenty-five strains of *Bt* and 10 strains of *C. rosea* were successfully isolated. The *Bt* colonies were identified by their circular, white, flat and undulate character, and the gram-positive and rod-shaped endospores. *C. rosea* was identified by white colonies on Potato-dextose agar and the characteristic conidiophores, which were branched and showed phialides at the tips.

In vitro screening of the isolates was undertaken to select the best isolates. The isolates that caused significantly greater mortality were *Bt* isolate B2, B10 and B12 and *C. rosea* isolates P1, P3 and P8. These isolates caused substantial nematode mortality in both faeces and water bioassay. Nematode counts were reduced by 28.5% to 62% and 44% to 69.9% in faecal bioassay for *Bt* and *C. rosea*, respectively. In the water bioassay, nematode counts were reduced by 62% to 85% for *Bt* and by 62.7% to 89.3% for *C. rosea*.

The best inoculum level at which the best isolates were most effective, and the optimum frequency of application were determined. The trial was conducted using bioassays with faeces and water. Inoculum levels of 10^6, 10^8, 10^{10}, 10^{12} spores ml^{-1} for *Bt* and 10^6, 10^8 and 10^{10} conidia ml^{-1} for *C. rosea* was used in the faecal bioassay. The inoculum levels tested in water bioassay were 10^6, 10^8, 10^{10} and 10^{12} spores ml^{-1} for *Bt* and 10^9, 10^{10}, 10^{11}, 10^{12} conidia ml^{-1} for *C. rosea*. In the faecal bioassay, B2 was the most effective *Bt* isolate at an inoculum level of 10^{10}

spores ml^{-1}. Isolate P3 was the best *C. rosea* isolate at 10^8 conidia ml^{-1}. In the water bioassay, Isolate P3 caused a mortality of 85% at inoculum levels of 10^9, 10^{10} and 10^{11} conidia ml^{-1}.

The performance of biological control agents in the field is sometimes inconsistent. Combining different biocontrol agents may be a method of improving their reliability and performance. However, the combination of most of the isolates was antagonistic, with efficacy less than that of either individual biocontrol agent. In particular, Isolate P3 was more effective when used alone than when combined with any other isolates. Therefore, the combination of biocontrol agents does not always result in synergistic interaction. There were some additive interactions between two bacterial isolates, and with one bacterial and fungal combination.

The effect of feeding the best of the biocontrol agents, or diatomaceous earth (DE), was evaluated in sheep. Two doses of *Bt* (1g and 2g kg^{-1}BW) and *C. rosea* (1g kg^{-1}BW) reduced the numbers of L3 nematode larvae in sheep faeces. The DE product (at 15% of feed) also reduced L3 numbers but it was less effective than either the *Bt* or the *C. rosea* products. Nematode counts were reduced by 74.6%, 75.1%, 84.6%, 68.5% and 27.5% for *Bt* 1g kg^{-1}BW, *Bt* 2g kg^{-1}BW, *C. rosea* (1g kg^{-1} BW), DE and control, respectively.

PREFACE

DECLARATION

I, **Mahlatse A. Baloyi**, declare that

i. The research reported in this thesis, except where otherwise indicated, is my original work.
ii. This thesis has not been submitted for any degree or examination at any other university.

iii. This thesis does not contain other personsô data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

iv. This thesis does not contain other personsô writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;
b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced.

v. Where I have reproduced a publication of which I am an author, co-author or editor, I have indicated in detail which part of the publication was actually written by myself alone and have fully referenced such publications.

vi. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections

Signed:é é é é é é é é é é .
Baloyi M.A. (Student)

Signed:é é é é é é é é é é .
Prof. Laing M.D. (Supervisor)

Signed:é é é é é é é é é é ..

Dr. Yobo K.S. (Co-supervisor)

ACKNOWLEDGEMENTS

First and foremost, I offer my sincerest gratitude to my supervisor, Prof. M.D. Laing, who has supported me throughout my study. I attribute the level of my masterôs degree to his encouragement and effort. One could not wish for a better and friendlier supervisor.

I owe my deepest gratitude to Dr. K.S. Yobo who has made endless support available in many ways as a co-supervisor.

I am grateful for the help and advice of Dr. M. Morris, and for providing formulations of *Bacillus thuringiensis* and *Clonostachys rosea* that were used in the field trial.

I thank Ms. Marion Young, Department of Animal Science, for assisting with the field trial design and the staff at Ukulinga Research Farm for providing sheep, the feedlot and technical assistance to run the trial.

I would like to express my gratitude to the students and staff in the Plant Pathology Department who supported me during the study.

I thank my mother, Mrs. Constance Baloyi, and my siblings, Tebogo, Nthabeleng and Allan, for their moral support and practical assistance in looking after my daughter, Marumo.

I am heartily thankful to my sponsors, the Ford Foundation International Fellowship Programme (Ford Foundation IFP) and the National Research Foundation (NRF), for the provision of financial support that made this study possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

TO MY WONDERFUL DAUGHTER

FOR ALL THE TIME I SPENT AWAY FROM YOU WHILE STUDYING

THESIS INTRODUCTION

Gastrointestinal nematodes of livestock have detrimental effects on animal health (Coles, 2005), causing financial losses due to the cost of treatments, reduced weight gain, and animal deaths (De and Sanyal, 2009). Consequently, farmers routinely use agrochemical anthelmintics. However, misuse and an over-reliance on these chemicals have resulted in the development of drug resistance to most classes of anthelmintics around the world (Condor et al., 1995; Waller, 1997; Van Wyk, 2001; Kaplan, 2004; De and Sanyal, 2009).

Several methods have been suggested to supplement or alternate with the use of these chemical anthelmintics. These include selective breeding for animals with greater nematode resistance (OõGrady, 2007), improved grazing management strategies (Barger, 1999), the use of condensed tannins (Min et al., 2004), use of the FAMACHA chart (**FA**fa **MA**lan **CHA**rt, which is the acronym for the originator of the idea, Dr. Faffa Malan) for the detection of anaemia levels (Van Wyk and Bath, 2002), vaccination, and biological control (Smith, 1997).

Biological control can be defined as the control of parasitic organisms by the use of naturally occurring antagonists to reduce the parasite populations to sub-economic levels. Research on *Duddingtonia flagrans* (Cooke) has shown that this fungus can be used successfully to control livestock nematodes (Larsen et al., 1995; De and Sanyal, 2009).

The research undertaken in this thesis followed a sequential series of trials following a logical progression from isolation, *in vitro* screening for efficacy, *in vitro* determination of dose levels, *in vitro* determination of frequency of application and speed of action, *in vitro* testing of combinations of the best biocontrol agents, and finally, *in vivo* field evaluation of the best agents. The overall goal was therefore to isolate several biocontrol agents from local soils and to identify the best isolates, doses, frequency of applications and to determine their performance in the field as biocontrol agents against livestock nematodes of small ruminants.

Thesis Structure

Chapter One is a review of the scientific literature covering the biology, epidemiology, economic importance and control options for livestock nematodes.

Chapter Two outlines the isolation of *Bt* and *C. rosea* from different habitats of the livestock section of Ukulinga Research Farm, University of KwaZulu-Natal, Pietermaritzburg.

Chapter Three covers the screening of different *Bt* and *C. rosea* isolates for biological control of nematodes.

Chapter Four reports on trials to identify the most effective concentrations of *Bt* and *C. rosea* and their interaction with time, for the control of sheep nematodes.

Chapter Five reports on trials using mixtures of *Bt* and *C. rosea* isolates against nematodes *in vitro*.

Chapter Six evaluates the effect of feeding of *Bt, C. rosea* and diatomaceous earth (DE) for control of sheep nematodes in an *in vivo* trial.

The Thesis Overview reviews the experimental results in terms of the original objectives and recommendations for future research.

References

Barger, I.A. 1999. The role of epidemiological knowledge and grazing management for helminth control in small ruminants. Int. J. Parasitol. 29, 41-48.

Coles, G.C. 2005. Anthelmintic resistance - looking to the future: a UK perspective. Res.Vet. Sci. 78, 99-108.

Condor, G.A., Campbell, W.C. 1995. Chemotherapy of nematode infections of veterinary importance, with special reference to drug resistance. Adv. Parasitol. 35, 1-84.

De, S., Sanyal, P.K. 2009. Biological control of helminth parasites by predatory fungi. Vetscan 4, 1-8.

Kaplan, R.M. 2004. Drug resistance in nematodes of veterinary importance: A status report. Trends Parasitol. 20, 477-581.

Larsen, M., Nansen, P., Wolstrup, J., Henriksen, S.A., Zom, A. 1995. Biological control of trichostrongyles in calves by hte fungus *Duddingtonia flagrans* fed to animal under natural grazing conditions. Vet. Parasitol. 60, 321-330.

Min, B.R., Pomroy, W.E., Hart, S.P., Sahlu, T. 2004. The effect of short-term consumption of a forage containing condensed tannins on gastrointestinal nematode parasite infections in grazing weather goats. Small Rumin. Res. 51, 279ï 283.

O'Grady, J., Akhurst, R.J., Kotze, A.C. 2007. The requirement for early exposure of *Haemonchus contortus* larvae to *Bacillus thuringiensis* for effective inhibition of larval development. Vet. Parasitol. 150, 97-103.

Smith, W.D. 1997. Prospects for vaccines against gastrointestinal helminth parasites of ruminants. In: Managing Anthelmintic Resistance in Endoparasites. A Workshop held at the 16th International Congress of the World Association for the Advancement of Veterinary Parasitology. (Eds.) Van Wyk, J.A., Van Schalkwyk, P.C. Sun City, Mafikeng, South Africa, pp. 6-12.

Van Wyk, J.A. 2001. Refugia- overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. Ond. J. Vet. Res. 68, 55-67.

Van Wyk, J.A., Bath, G.F. 2002. The FAMACHA system for managing haemonchosis in sheep and goats by clinically identifying individual animals for treatment. Vet. Res. 33, 509-529.

Waller, P.J. 1997. Sustainable helminth control of ruminants in developing countries. Vet. Parasitol. 71, 195-207.

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Many parasitic protozoa and helminths inhabiting the alimentary tract of grazing ruminants and horses worldwide are of veterinary importance because their parasitism causes structural and functional changes in the digestive physiology of the host (Hoste, 2001). Gastrointestinal nematodes (roundworms) (GIN) are the most damaging internal parasites (Waller, 1997; Vercruysse and Clarebout, 2001). In a survey by Perry et al. (2002), investigating the priorities for health of livestock among smallholder livestock keepers in Africa and Asia, GIN infections were identified as being the most serious problem. Vatta et al. (2001) identified *Haemonchus contortus* (Rudolphi) as the single most important constraint to the production of small ruminants on both commercial farms and in resource-poor farming systems in South Africa. Gray (1993) classified the principal livestock nematodes according to their location in the host, which included those that attack the abomasum: *Haemonchus* spp. [*H. contortus* (sheep, goat, and young calves), *H. placei* (Place) (cattle)], *Ostertagia* spp., [*O. ostertagi* (Stiles) (cattle), *O. circumcincta* (Stadelmann) (sheep, goat)], *Trichostrongylus axei* (Cobbold) (ruminants and horses); those that attack the small intestine; (*Trichostrongylus* spp., *Cooperia* spp., *Nematodirus* spp.); and those that attack the lung; *Dictyocaulus* spp., [*D. viviparous* (Bloch) (cattle) and *D. filarial* (Rudolphi) (sheep, goat)]. Host susceptibility varies as a function of age, vigour, genetic constitution, presence or absence of an already established infection, and in some instances, acquired immunity (Georgi, 1969).

Mitreva et al. (2007) associated the infections by these pathogens with extensive suffering in veterinary animals, resulting in major losses in livestock production, due to disease and the cost of implementing control programs. In Australia McLeon (1995) estimated a loss of about $222M per annum (Table 1.1), as a result of control of livestock internal parasites via chemicals and

labour, and production losses (loss of meat and wool to disease and death of infested animals). Of all the species of gastrointestinal nematodes commonly found in livestock, the Trichostrongylus species, *Haemonchus contortus* and *Teladorsagia circumcincta* (Giles), are the most abundant and cause the greatest losses in livestock production, especially in sheep (O'Connor et al., 2006).

Table 1.1 Cost of livestock intestinal parasites chemical control in some countries

Livestock	**Country**	**Losses p.a. (US$)**	**References**
Sheep	Australia	$222M	Mcleon, 1995
Sheep	Denmark	$10M	McLeon, 1995
Dairy cattle	Netherland	$120M	Corwin, 1997
Sheep	Australia	$300M	Corwin, 1997
Sheep and goats	Kenya	$26M	Maichomo et al., 2004

1.1.1 Diagnosis and immune response of livestock nematodes

Trichostrongyle infection can be diagnosed by examination of the faeces for parasitic eggs (McKenna, 1981). Eggs can be found in the faeces about 2 to 3 weeks post-infection and faecal culturing of the worm carried in the gut of sheep and goat. With Type I diseases there are usually more than 1 000 eggs per gram of faeces. With Type II diseases, the egg count is highly variable, may be negative and is less useful. In young animals, an initial diagnosis of GIN infestation may be made as a result of the clinical signs of loss of appetite, weight loss and diarrhoea (Urquhart et al., 1987). At the cellular level, *Haemonchus* spp. produce a response that is characterized by an increased number of globular leucocytes (mucosal mast cells that have released their granules), mucosal mast cells, and eosinophils in the abomasal mucosa. Resistant sheep have more of these cells when compared to susceptible sheep. The eosinophils and globular leucocytes are responsible for the expulsion of the GIN from the gut, preventing establishment of the parasites.

1.1.2 Climatic distribution and epidemiology

Development and survival of the infective stage depends on the prevailing conditions of temperature and moisture. Optimum requirements vary distinctly among GIN species (Georgi, 1969). In South Africa, *O. circumcincta* is the dominant parasite in winter and in uniform rainfall areas, with their main survival advantage being a greater resistance to drought and the ability to develop at lower temperatures than *H. contortus* (O'Connor et al., 2006). *Haemonchus contortus* is the most important nematode species of sheep in tropical and subtropical areas, or regions with summer-dominant rainfall. *H. contortus* becomes less significant as the climate tends towards winter (O'Connor et al., 2006). The success rate and speed of development of gastrointestinal nematodes are determined mostly by temperature and moisture, both of which have major influences on the epidemiology of the nematodes (Chatterjee et al., 2007). According to Stromberg (1997), temperature controls the rate of development, but without moisture, there will be no change in the growth rate of the nematodes. Furthermore, temperature may affect viability and/or infectivity of larvae. The movement of nematode larvae is facilitated by a continuous film of moisture on herbage, as a result of irrigation, rainfall or dew. When moisture is not a limiting factor, temperature asserts a greater influence over migration (Stromberg, 1997). *Trichostrongylus circumcincta* and *T. colubriformis* develop at lower temperatures than *H. contortus*. Hsu and Levine, (1977) observed that at 20^0C and 25^0C, eggs of *H. contortus* and *T. colubriformis* took a minimum of 4 and 3 days respectively to reach the L3 larval stage. O'Connor et al. (2006) stated that once development to the L3 stages is complete, all three major species are less susceptible to unfavourable climatic conditions. They further suggested that this could be attributed in some part to the larval ability to migrate to more favourable micro-environments. Hiding in the sheath of the previous larval stage prevents feeding of infective larvae, and makes them susceptible to higher temperatures which increase their metabolic rate through depletion of energy reserves. Research in Ethiopia indicated that there was a distinct seasonal increase in the availability of larvae on pastures in response to climatic changes (Tembely et al., 1997). In Tanzania, faecal egg count (FEC) increased during the rainy season (December-April) (Keyyu et al., 2005).

1.1.3 Economic importance of livestock nematodes

There is a worldwide awareness that GIN infections are one of the major causes of loss of productivity in grazing livestock (Perry et al., 1999). Infestation of livestock with high levels of GIN detrimentally affects the profitability of invested capital in terms of animal health and performance (Corwin, 1997). This includes weight losses due to reduced feed intake, reduced carcass quality and reduced wool production and quality (Gray, 1993). To measure the effects of subclinical or economic thresholds of GIN parasitism, measurement of performance parameters such as weight gain, feed conversion, forage utilization, conception rate, calving-breeding interval, milk production and disease resistance are employed (Bumgarner et al., 1986; Williams et al., 1986; Wohlgemuth et al., 1990; Gibbs, 1992; Toombs and Craig, 1994 *cited by* Corwin, 1997). Economic losses are primarily due to mortality, but can also result from subclinical effects such as inadequate weight gain, feed consumption, and reproductive potential, as well as reduced lactation, wool and meat production due to infections by *Oesophagostomum* spp. Reports indicate that mortality in sheep flocks may exceed 40%, while weight losses of 6-12 kg/year/animal may occur (IEMVT, 1980 *cited by* Krattiger, 1997). However, decreased efficiency in feed utilization associated with clinical or chronic conditions is often the main cause of economic losses (Holmes, 1987). About US$ 800 million p.a. were estimated to be spent on chemical treatments to control helminths (Krattiger, 1997). Because nematodes such as *H. contortus* are blood feeders, heavy infestations in animals can cause severe anaemia and death. Effects of other nematodes, such as those from the genus *Cooperia* and *Trichuris* spp., are in addition to those of infestation with other nematodes.

1.1.4 Life cycle of livestock nematodes

Most livestock nematodes undergo the following lifecycle, according to Urquhart et al. (1987). The cycle begins when the mature female lays eggs which are passed with animal faeces to the ground. Under favourable conditions, these eggs hatch into the first larval stage (L1). The larvae feed, grow and moult in the faeces. After a first moult, the larvae develops into the second larval stage (L2) which grows, feeds and eventually undergoes the second moult. In most *Strongylida*

nematode species, the sheath of the second moult is retained, thus providing an enclosed space for the development of the third larval stage (L3), which does not feed. This is the infective stage, and the L3 stage is typically eaten by livestock, along with pasture grass on which the L3 stage sits. After entering the animal host, the larvae removes the sheath of the second moult and is then referred to as the third parasitic stage (P3). At this stage the nematode feeds on the animal host and grows until it moults for the third time, after which the development of the sexual organs starts. In the fourth stage, the sexual organs continue to develop, and males and females are easily identified for the first time. The parasites feed and grow considerably before undergoing the fourth moult. Growth and sexual development continues up to the fifth stage. True adults are only present after mating.

Table 1.2 Summary of livestock nematodes from Oslon (1974); and Swartson and Nsahlai (2006)

Nematode Genus	**Morphology**	**Life cycle**	**Symptoms**	**Common name**
Haemonchus contortus	Males are 10-20 mm and females 18-30 mm long. White uteri and ovaries; wind around the blood-filled intestine in a barber-pole appearance	Direct (does not require intermediate host).	Acute anaemia, severe blood loss, bottle jaw, diarrhoea, pale gums and inside of the eyelids (conjunctiva).	Barber-pole
Nematodirus spp.	10-30 mm long, with thin anterior and swollen appearance of the head.	Direct. 15-28 days.	Loss of appetite, diarrhoea, weight loss and reduced wool production.	Thread necked strongyle

Nematode Genus	Morphology	Life cycle	Symptoms	Common name
Trichostongylus spp.		Direct. Prepatient period of 20- 25 days.	Severe weight loss, poor growth, wart-like swellings, diarrhoea and reduced appetite	Bankrupt worm or stomach hair worm
Cooperia spp.	Brownish-red, 4-6 mm in length with swelled anterior	Direct. Prepatient period of 15-20 days	Loss of appetite, diarrhoea and weight loss.	Small intestine worm
Oesophagostomum spp.	Up to 20 mm long, with a narrowed front.	Direct. 6-7 days.	Diarrhoea, thickening of large intestinal wall, mucus production in faeces,	Nodular worm
Trichuris spp.	Have slender neck and a thick hind-end, males are 50-80 mm with a curved tail. Females are 35-70 mm.	Prepatient period of 1-3 months.	Thickening of the caecal wall and diarrhoea.	Whipworm

1.2 Control of livestock nematodes

Extensive experience exists in the control of parasitic nematodes that infect agricultural and companion animals. Methods of nematode control are diverse and have focused on control rather than eradication (Waller and Thambsborg, 2004; Sayers Sweeney, 2005; Stear et al., 2006). This is because of continuous to long-lived parasites on pastures and incomplete resistance responses of the host.

1.2.1 Chemical control of livestock nematodes

According to Mitreva et al. (2007), the use of anthelmintics is still the core strategy for nematode control. Their success has been cyclical and directly related to the timely introduction of new drugs as resistance to older drugs has surfaced. Anthelmintics are grouped into four main chemical classes, according to their modes of action (Stepek et al., 2004).

1.2.1.1 The benzimidazoles class

The benzimidazoles (e.g. albendazole) are a large chemical family used to treat nematode and trematode infections in domestic animals. This group disrupts ϐ-tubulin synthesis (Stepek, et al*.*, 2004). However, with the widespread development of resistance and the availability of more efficient compounds which are easier to administer, their use has rapidly decreased. In ruminants, treatment with the benzimidazoles removes most of the major adult gastrointestinal (GI) parasites and many of the larval stages. Metabolism and excretion of benzimidazole compounds is more extensive in cattle than in sheep. Consequently, the systemic anthelmintic activity of most benzimidazoles is greater in sheep than in cattle, and dose rates in cattle are often higher than those in sheep. Albendazole, fenbendazole, oxfendazole, and febantel are active against inhibited fourth-stage larvae of *Ostertagia spp.*; however, inconsistent efficacy has been reported. Efficacy against *D. viviparus* has also been noted for these insoluble benzimidazoles. Oxfendazole, albendazole, and febantel are minimally teratogenic in sheep, whereas fenbendazole, mebendazole, and oxibendazole are not. An oxfendazole pulse-release bolus for intraruminal use has been developed for cattle whereby 5 therapeutic doses of oxfendazole (750 or 1,250 mg / tablet) are released approximately every three weeks in the rumen. An albendazole slow-release capsule containing 3.85 g albendazole and delivering a daily dose of 36.7g for 107 days has been marketed for small ruminants (Merck and Co., Inc., 2008).

Albendazole and netobimin at 20 mg kg^{-1} are active against mature *F. hepatica*. The other benzimidazoles and probenzimidazoles used for nematode control have only a marginal efficacy against liver flukes (Merck and Co., Inc., 2008). Because of the lack of efficacy against the immature stages, most benzimidazoles are not indicated for treatment of acute fascioliosis (Merck and Co., Inc., 2008).

The anthelmintic activity of tetramisole, a racemic mixture, lies in the l-isomer, levamisole. It is commonly used in cattle, sheep, pigs, goats and poultry to treat nematode infections; however it has no activity against flukes and tapeworms. The efficacy is generally considered equivalent with either product (Merck and Co., Inc., 2008).

1.2.1.2 Imidazothiazole class

Levamisole acts on the roundworm nervous system and is not ovicidal. Its broad spectrum of activity, ease-of-use (being water soluble), reasonable safety margin, and lack of teratogenic effects have allowed it to be used successfully. Because of its mechanism of action, the peak blood concentration is more relevant to its antiparasitic activity than the duration of concentration. Levamisole resistance appears to be associated with a loss of cholinergic receptors. Levamisole has immunostimulant effects at dosage rates higher than those used for anthelmintic activity, and it has been used in humans and, to a limited extent, in other animals against several diseases (Merck and Co., Inc., 2008).

In ruminants, levamisole is highly effective against the common adult GI nematodes and lungworms, and many larval stages. It lacks efficacy against arrested larvae, such as those of *Ostertagia ostertagi*. Slow-release boluses of levamisole are available in some countries and contain 22.05 mg levamisole. They release 2.5 mg during the first 24 hours and the remainder over a 90-day period (Merck and Co., Inc., 2008).

Pyrantel was first introduced as a broad-spectrum anthelmintic against GIN of sheep and has also been used in cattle, horses, dogs and pigs. It is available as a citrate, tartrate, embonate, or pamoate salt. Aqueous solutions are subject to isomerization on exposure to light, with a resultant loss in potency; therefore, suspensions should be kept out of direct sunlight. It is not recommended for use in severely debilitated animals because of its levamisole-type pharmacological action.

Pyrantel is used as a suspension, paste, drench, or tablets. Both pyrantel and morantel are effective against adult gut worms and larval stages that dwell in the lumen or on the mucosal surface. Pyrantel tartrate is effective as a broad-spectrum anthelmintic in ruminants; however, its activity is mainly limited to the adult GI nematodes (Merck and Co., Inc., 2008).

1.2.1.3 Macrocyclic lactones class

The macrocyclic lactones (avermectins and milbemycins) are chemical by-products from soil microorganisms of the genus *Streptomyces*. The macrocyclic lactones are highly lipophilic anthelmintics which are known to bind to ligand-gated ion channels, opening them (Prichard and

Roulet, 2007). As a result, they have a potent, broad-spectrum antiparasitic activity at low dose levels (Merck and Co., Inc., 2008). They are active against many immature nematodes and arthropods. However, the commercially available products are primarily excreted in the faeces and kill non-target dung insects, therefore affecting dung dispersal. Decomposition of invermectin in faeces or soil is slow, especially in winter (Merck and Co., Inc., 2008).

1.2.1.4 Amino-acetonitrile derivatives class (AAD)

This is a new class of chemicals with a novel mode of action involving a unique, nematode-specific clade of acetylcholine receptor subunits. They are well tolerated and of low toxicity to mammals, and overcome existing resistance to the currently available anthelmintics. The AAD are low molecular mass compounds that are easily accessible by alkylation of phenols with chloroacetone. When tested in ruminants, all AADs were able to eliminate fourth larval (L4) stages of *H. contortus* in sheep and *Cooperia oncophora* (Raiilet) in cattle with a single oral dose of 20 mg (Malan et al., 2001).

1.2.2 Mechanism of action

Anthelmintics inhibit metabolic processes that are vital to the parasite but not vital to, or absent in, the host. Alternatively, they have inherent pharmacokinetic properties that cause the parasite to be exposed to higher concentrations of the anthelmintic than the host cells. While the exact mode of action of many anthelmintics is not fully documented, the sites of action and biochemical mechanisms of many of them are known. Parasitic helminths must preserve an appropriate feeding site, and nematodes must actively ingest and move food through their digestive tracts to maintain an appropriate energy state. Feeding and reproductive processes require proper neuromuscular coordination. Parasites must also maintain homeostasis despite host immune reactions. The pharmacological basis of treatments against helminths generally involves interference with the integrity of parasite cells, neuromuscular coordination, or protective mechanisms against host immunity, which leads to starvation, paralysis, and expulsion of the parasite (Merck and Co., Inc., 2008).

1.2.3 Problems associated with chemical control
-Multi-drug resistance

Using only a chemical control strategy has numerous problems. Among these is the overuse or under-dosing of chemotherapeutic drugs, which results in resistance to these drugs (Geerts and Greyseels, 2000; De and Sanyal, 2009). Occurrences of internal parasite populations resistant to available anthelmintics have increased. Resistance has been reported to drugs within three major classes of anthelmintics (Terrill et al*.,* 2001). Anthelmintic resistance of gastrointestinal nematodes of livestock is most prevalent in Australia, New Zealand, South America and South Africa (Van Wyk, 1990; Coles et al., 1994; Van Wyk et al., 1999). The initial reports of anthelmintic resistance were to phenothiazine, reported in the late 1950s and early 1960s. Drudge (1957) reported the first incidence of drug resistance with *H. contortus* in sheep. This was followed by reports of resistance by cyathostomins (small strongyles) of horses (Poynter and Hughes, 1958; Gibson, 1960) (Table 1.3). In 1961, thiabendazole was introduced as the first anthelmintic that provided a broad-spectrum nematicidal activity coupled with low mammalian toxicity. The rapid acceptance and widespread use of thiabendazole and other benzimidazole anthelmintics resulted in resistance to this class evolving within a few years, first by the sheep nematode, *H. contortus* (Conway, 1964; Drudge et al., 1964), followed by equine cyathostomins. Benzimidazole resistance was later reported in other major gastrointestinal trichostrongylid nematodes of sheep, namely *Teladorsagia* (*Ostertagia*) *circumcincta* (brown stomach worm) and *Trichostrongylus colubriformis* (black scour worm). By the mid-1970s, studies showed common and widespread incidences of resistance to the benzimidazoles by a wide range of nematodes. Similarly, resistance to the imidazothiazoleï tetrahydropyrimidine and avermectinï milbemycin classes of anthelmintics was observed after their introduction. Most alarmingly, by the early 1980s, reports of multiple-drug resistant (MDR) worms appeared for the first time (Coles, 1986).

-Other related problems of anthelmintics

In addition to anthelmintic resistance, increasing public awareness regarding contamination of food with chemicals has resulted in consumer pressure to reduce drug residues in meat and meat products. This influences the use of anthelmintics (Larsen, 2000). Levamisole and the benzimidazoles pose little cause for concern, but there is greater worry regarding the avemectins (De and Sanyal, 2009). Ivermectin is excreted in faeces in sufficient quantity to have a detrimental effect on invertebrates that usually degrade dung heaps, and hence on organisms higher up the food chain (Cox, 1999; De and Sanyal, 2009). Anthelmintics are relatively expensive for smallholder farmers in relation to available resources, reducing the usefulness of these drugs in developing countries (Knox, 2000; Dalton et al., 2001).

Table 1.3: Worldwide levels of anthelmintic resistance among livestock hosts (as outlined by Drudge and Elem, 1964, and subsequently by Van Wyk et al. (1999) and Coles et al. (1994))

Drug class	Hosts with high levels of resistant GIN	Hosts with emerging resistance in GIN	Major livestock-producing areas where drug is still highly effective in sheep, goats and horses
Benzimidazoles	Sheep, goats, horses	Cattle	None
Imidothiazoles			
Levamisole (ruminants)	Sheep, goats	Cattle	None
Pyrantel (horses)	Horses (USA only)	Horses	Unknown ï few recent studies outside USA
Avermectin			
Ivermectin	Sheep, goats, cattle	Cattle, horses	Horses ï worldwide
			Sheep, goats ï Europe, Canada
Moxidectin	Goats	Sheep, goats, cattle, horses	Horses ï worldwide
			Sheep ï most regions

1.2.4 Alternative control methods

1.2.4.1 Botanicals

An alternative anthelmintic strategy with considerable potential is the use of extracts from medicinal plants (Stepek et al., 2004). A wide range of plants and plant extracts has been used traditionally for the treatment of helminth infections of humans (Waller et al., 2001). Many attempts have been made to evaluate the plant extracts with anthelmintic potential for the control of livestock parasitic nematodes (Table 1.4 and 1.5).

Table 1.4: Some botanicals used to control livestock nematodes

Plant name	**Country of Use**	**Method of Preparation**	**Comments**	**References**
Myrsine africana	Kenya	Dried fruits and leaves	Ineffective even with the highest dose	Githiori et al., 2002.
Seracia lepedeza	USA	Dried ground hay	Effective reduction of nematodes	Min and Hart, 2003, Min et al., 2004.
Papaya carici	Not stated	Latex extract	Effective	Stepek et al., 2004.
Ficus spp.	Not stated	Juice extract	Effective	Stepek et al., 2004.
Ananas comosus (pineapple)	Not stated	Juice extract	Effective	Stepek et al., 2004
Ananas comosus	Pakistan and Philippines	Raw powder	Ineffective	Hördegen et al., 2003.
Azadirachta indica	Pakistan and Philippines	Powder or methanol extract	Effective	Hördegen et al., 2003.
Spondias mombin	Nigeria	Aqueous and ethanolic crude extract	Effective	Ademola et al., 2005
Hedysarum coronarium	USA	Condensed tannin extracts in aqueous solution	Effective	Nienzen et al., 2002.
Lotus pedunculatus	USA	Condensed tannin extracts in aqueous solution	Effective	Molan et al., 2000

Plant name	Country of Use	Method of Preparation	Comments	References
Ornobrychis viciafolia	USA		Effective	Paolini et al., 2003
Caesalpinia crista	Pakistan and Philippines	Aqueous ethanol extract	Ineffective	Hördegen et al., 2003.
Vernonia anthelmintica	Pakistan and Philippines	Raw powder	Ineffective	Hördegen et al., 2003.
Fumaria parviflora	Pakistan and Philippines	Aqueous ethanolic extract	Ineffective	Hördegen et al., 2003.
Albizia anthelmintica	East Africa, Kenya, Sweden, Ethiopia, Uganda	Heat treated or soaked in water	Ineffective	Minja, 1994; Grade and Longok, 2002; Desta, 1995.

Sources and modes of action of botanicals to control helminths

Upon injury, some plants such as papaya and fig trees are known to produce latex which is rich in proteolytic enzymes, whereas other plants such as pineapple contains large amounts of cysteine proteinases in the juice extracted from the stem or fruit. These enzymes have many medicinal uses. Robbin (1930), *cited by* Stepek et al. (2004), reported that the active anthelmintic principle of *Ficus spp*., named ficin, was an enzyme that damaged the cuticle of *Ascaris suum,* presumably by proteolytic digestion. Fresh pineapple juice was found to possess an enzyme, bromelain, which is similar to ficin. A study by Stepek et al. (2004) showed that proteolytic enzymes from the genus *Ficus* have been most closely associated with botanical anthelmintic action.

Min et and Hart (2003) reported another plant-based GIN control method, namely the grazing or feeding of livestock with plants containing condensed tannins (CT). Growing these plants for hay or grazing, to be used as a natural de-worming agent, may be a cost-effective, environmentally

friendly alternative to the exclusive use of chemical anthelmintics by small ruminant producers (Shaik et al., 2004).

Table 1.5 Alternative strategies for controlling gastrointestinal nematodes of livestock

Control options	Methods and effectiveness	Comments	References
Genetic resistance breeding	By the use of natural diversity of the host genome to reduce parasite transmission	In cows the number of nematode eggs/gram of faeces was influenced by the host genetics, with an estimated heritability of 0.30. A small percentage of the herd was responsible for the majority of parasite transmission, suggesting that genetic selection could reduce the overall parasite transmission. However, this is still far from being widely implemented.	**Leighton et al., 1989; Gasbarre et al., 1990; De and Sanyal, 2009**
Vaccination	Protection is induced with natural antigens derived from the intestine of the nematode. Used successfully against *Haemonchus contortus;* other parasites still present challenges to identify good vaccine candidates	Deeper understanding of both the important antigenic characteristics and mechanisms utilized by nematodes to survive host immune responses is indispensable. Despite promising results and mass appraisal over the years, a commercial product is yet to be released.	**Knox and Smith, 2001 *cited by* Mitreva et al., 2007; De and Sanyal, 2009**
Grazing management	This includes field rotation and rotation between different animal species.	This is expensive because it requires enough land and animals	**Anthanasiadou et al., 2001 and Stepek et al. (2004).**
Control options	**Methods and Effectiveness**	**Comments**	**References**
Use of botanicals	The use of plants that have anthelmintic properties.	As seen in Table 1.5, some plants are effective, although some ñremediesò are poisonous or ineffective.	
Use of copper oxide wire	This was initially used for copper deficiency in sheep	Copper oxide wire particles did not seem to be effective in controlling L4 stage of *H.*	**Burke et al., 2007**

		contortus which also feed on blood of livestock. Although results are promising at higher doses they may lead to copper toxicity.	
FAMACHA©	A novel clinical test developed to assess the level of anaemia caused by haemonchosis in sheep and to determine which animals will receive anthelmintic treatments	The product has shown to minimise the incidence of chemical resistance by dosing sheep at the right time	**Malan et al., 2001**
Uses of biocontrol agents	**Use of microorganisms to control gastrointestinal nematodes**	**Biological control on pasture includes the use of predatory fungus and bacteria to kill a variety of nematode species and substantially reduce the intensity of infection. Promising candidates are *D. flagrans, C. rosea* and *Bt***	**Larsen, 1999 and O'Grady et al., 2007**

1.2.4.2 Biological control

Over the last 10-15 years, biological control has been identified as a viable alternative to chemical anthelmintics (De and Sanyal, 2009). Biological control may be defined as the use of living microorganisms introduced into an environment to control a target microorganism and thereby reduce the population growth of the target to a threshold below which it no longer causes clinical problems and economic losses (Larsen, 2006).

Waller and Faedo (1996) explained that biological control agents may not only include the classically exploited organisms, but also that these organisms may be genetically modified to enhance their properties. Hence biological control may be divided into two major categories: natural and applied. Natural biological control is influenced by native or co-evolved natural enemies in the environment without human intervention. Applied biological control involves the repeated application of cultured biological control agents (De and Sanyal, 2009)

Waller (1997) reported that biocontrol agents target the free-living larval stage of the nematode rather than the parasitic stages in the host. This approach focuses on the faecal deposits in which eggs, and the L1 and L2 larval stages live. According to De and Sanyal (2009), biocontrol agents do not totally eliminate target organisms, but reduce their numbers to acceptable levels, and maintain a population balance between the pathogen and the antagonist. Therefore, the goal of using a biocontrol approach is to lower the numbers of parasitic populations to below the clinical level, aiming to go below the economic threshold above which production losses occur. According to Faedo and Krecek (2002), when considering the control of helminths in livestock, it is important to abandon the unrealistic concept of eradication which has led to an over-reliance on anthelmintic drugs, resulting in drug resistance within the target population.

Githiori et al. (2002) mentioned that although anthelmintic drugs have long been considered effective, they are expensive and sometimes unavailable to smallholder farmers and pastoralists in developing countries. Thus biological control has been considered as an alternative control method for small-scale livestock farmers.

Biological control of parasitic nematodes in livestock therefore aims to establish a situation where the grazing animals are exposed to a low level of infective larvae, at which naturally acquired immunity will develop in the animals (Thamsborg et al., 1999).

One of the most important aspects in biocontrol programmes is the accurate identification of the pest and any hyperparasitic organisms that might control that pest. This aspect has a direct impact not only on determining the geographic range of the pest and its hyperparasites, but also on the acquisition of permits necessary for the release of biocontrol agents (Schnepf et al., 1998). Biological control has the capacity to reduce a range of nematode parasites, not only within but also between species of livestock (Waller, 1997). Fontenot et al. (2003) reported an overall reduction of a wide range of nematodes, as a result of the application of biocontrol agents. Furthermore, biocontrol agents are chemical-free, thus producers can capitalise on the residue-free, organic meat products that are in high demand from consumers and command a premium price (Waller, 1997). However, because *B. thuringiensis* is increasingly used in agricultural pest management, this has resulted in the occurrence of resistance in a cruciferous pest, diamond-

backed moth (*Plutella xylostella)* (Navon, 2000). This is a recognized problem if *Bt* strains with a single Cry toxin are used, and reflects the need to manage resistance, even with biocontrol agents.

Several methods of incorporating this agent into the gut of target animals have been tested and, so far, daily feeding of the spores or cells of the biocontrol agents, together with supplements, or in the use of slow release devices such as mineral blocks, pellets or boluses, seem to be the most successful (Waller, 1997; Fontenot et al., 2003). For biocontrol agents such as *B. thuringiensis,* delivery of viable spores into the intestinal tract of the sheep is a challenge. Studies by Wu and Papas (1997) showed that application of reverse enteric coating techniques allowed for the passage of the formulated material through the rumen, followed by release into the abomasum because it was stimulated by entry into an acidic environment. This emphasised the need to protect the spores from the acidic conditions in the faeces. Shimoda et al. (2001) and Senel and McClure (2004), discovered that the use of the adhesive properties of chitosan ensured that *Bt* was localised at the site of the nematode.

The following requirements need to be met by a commercial biocontrol product for use in an integrated control strategy: the product must be (i) cheap to produce, (ii) safe to handle, (iii) survive passage through the gastrointestinal tract and (iv) be able to grow and subsequently germinate in the voided dung, in order to kill the eggs and developing larvae (Larsen et al., 1991).

Duddingtonia flagrans as a biocontrol agent

Many fungi exhibit anti-nematode properties, characterized by their ability to capture and exploit nematodes either as a primary source of nutrients or as a supplement to a saprophytic existence. These fungi are divided into predacious, endo-parasitic and egg-destroying fungi (Faedo and Krecek, 2002).

Duddingtonia flagrans (Cooke) has been shown to have considerable potential as a biocontrol agent, and may be commercialized in the near future, given that the basic research on the fungus has been completed. *D. flagrans* was selected based on its ability to survive passage through the gastrointestinal tract of ruminants and subsequently to trap the developing parasite larvae in the deposited faeces (Larsen et al., 1994). This species (formerly *Trichothecium flagrans*) belongs to

the Family Deuteromycetes, which are the members of the Fungi Imperfecti, of which a number are known as nematode destroying fungi (Cooke et al., 1964; Barron, 1977). *D. flagrans* has a particular characteristic of producing abundant intercalary chlamydospores (Cooke, 1969). Previous studies in Denmark by Larsen and co-workers on the ability of nematophagous fungi to survive the environmental rigours of gut passage in cattle, showed better survival of *D. flagrans* than the other well-known nematophagous genus, *Arthrobotrys*, under both *in vitro* (Larsen et al., 1991) and *in vivo* (Larsen et al., 1992) conditions.

According to Larsen et al. (1991), the special feature that ranks *D. flagrans* above other nematophagous-fungi is its production of many resistant, thick-walled resting spores. These survive the unfavourable conditions in the gut. This property allows chlamydospores to be administered orally, resulting in their deposition in dung simultaneously with the eggs of internal parasites. In warm dung the parasite eggs hatch to release free-living, bacterial-feeding, juvenile stages, while the chlamydospores germinate to form mycelia that produce traps in response to nematode activity.

Daily feeding of *D. flagrans* spores to grazing animals for 3-4 months prevents the build up of dangerous levels of infective GIN larvae on pasture. Knox et al. (2001) found that sheep that were fed a supplement containing *D. flagrans* chlamydospores had lower egg counts and improved live weight gains compared to untreated animals. A study by Fontenot et al. (2003) showed that daily feeding of 5×10^5 chlamydospores per kg of body weight of *D. flagrans* resulted in a reduction of GIN larvae in faeces, which subsequently resulted in lower pasture infectivity and reduced nematode burdens in tracer animals. According to Larsen et al. (1998) and Peña et al. (2002), this dosage of 5×10^5 chlamydospores per kg of body weight is adequate to produce substantial nematode trapping. Reports by Larsen et al. (1991), Gronvold et al. (1993), Mendozade de Gives et al. (1998) and Peña et al. (2002), estimated that routine oral doses of *D. flagrans* resulted in a reduction of GIN L3 larval stage counts in faecal culture of 30% to 90%, with an 85% reduction being the average.

Challenges to the use of nematode trapping fungi in the control of nematodes have been the requirement for daily administration of the fungus to the host, and achieving the required fungal density inside the dung. However, an isolate of *D. flagrans* from New Zealand (Skipp et al.,

2002) demonstrated a trapping efficiency of 78% and activity for up to 90 days on pasture (Waghorn et al., 2003).

The interest in studying the infection biology of these fungi comes from their potential use as biological control agents against plant and animal parasitic nematodes (Tunlid et al., 1999). Utilization of nematode-trapping fungi for agriculture could be a powerful tool for biological control of plant-feeding nematodes on farms (Stirling et al., 1998).

Mechanism of action of *D. flagrans*

D. flagrans targets the free-living larval stages outside the host. These stages exist as larvae within the faecal deposit, and as infective third-stage larvae on pastures. The best way to apply the fungus to the faecal environment is via passage through the gastrointestinal tract of the host. *D. flagrans* is unique in that it produces large numbers of chlamydospores which, when fed to livestock, can survive passage through the gastrointestinal tract to be deposited in their faeces. Once voided in the faeces, *D. flagrans* grows within the faecal deposit and traps emerging larvae. While developing, the fungus produces three-dimensional mycelia ñnetsò that trap migrating nematodes and thus greatly reduce parasitic larval development (Larsen, 2000). This prevents the L3 larvae from developing (Faedo et al., 2002). However, nematophagous fungi such as *D. flagrans* do not affect the established populations of nematodes within the host and are thus not curative (Faedo et al., 2002).

***Clonostachys rosea* as a biological control agent**

Clonostachys rosea (Schroers) was previously known as *Gliocladium roseum* (Bainier) classified under the phylum Ascomycota and family Bionectriaceae (Schroers et al., 1999). This fungus is an important biological control agent of plant fungi and nematodes (Huang et al., 2009). Dong et al. (2004) reported that some toxin isolated from filtrate of *C. rosea* showed strong nematicidal activities against *Caenorhabditis elegans* (Maupas). *C. rosea* is capable of withstanding harsh environmental conditions (Morandi et al., 2003). It has mostly been isolated from cultivated grassland and woodlands, forests, heathland, freshwater and coastal soil of neutral to alkaline pH soils (Sutton et al., 1997). *C. rosea* is mostly found in temperate and tropic regions. *C. rosea* is a

motoscopic fungi used as phytopathogenic fungi (Li et al., 2002), and a facultative parasite of pathogenic nematodes (Yang et al., 2000). When *C. rosea* was applied to strawberry on a weekly basis, numbers of rotten fruits were reduced by 73% and were similar to the control provided by application of fungicide (Valdeberito-Sanhueza et al., 1997).

Mode of action of *C. rosea*

According to Zhang et al. (2008), pathogenesis occurs when the conidia adhere to nematode cuticle and germinates, then produces a germ tube that penetrate the host body and kill it. The mycoparasitic activity has been attributed to the secretion of its cell-wall degrading enzymes capable of degrading chitin. Zhao et al. (2005) and Li et al. (2006) reported two extracellular serine proteases (PrC and Lmzl) from *C. rosea* which were both found to play a role in nematode infection. *C. rosea* Isolate 87 from soil sample showed capability of parasitizing and digesting nematodes (Zhang and Zhao et al., 2004).

***Bacillus thuringiensis* as a biocontrol agent**

Bacillus thuringiensis (Bt) (Berliner) is a gram-positive, spore-forming bacterium which has the unusual property of producing a parasporal protein crystal (delta-endotoxin) which is toxic (*Cry* proteins) to some insect pests (Lecadet et al., 1999, *cited by* Quesada-Moraga et al*.,* 2004). Swadener (1994) described *B. thuringiensis* as a species of bacterium that has insecticidal properties affecting a selective range of insect orders. Swadener (1994) further explained that there are at least 34 subspecies of *B. thuringiensis* (also called serotypes or varieties). There are already more than 800 strain isolates (De Barjac and Franchon, 1990). *B. thuringiensis* products have captured 90-95% per cent of the biopesticide market because of their unique properties and advantages as biopesticides (Entwistle et al., 1993). One of *Bt*ôs most desirable characteristics is its selectivity, due to the selectivity of the protein crystals. However, this is complemented by the many strains available that attack a wide range of specific target pests.

B. thuringiensis is fermentation-friendly and therefore it is relatively easy to exploit commercially. Furthermore, *Bt* is a prokaryote, meaning it does not have dominant or recessive alleles, thus making it relatively easy to manipulate genetically. The gene for the crystal protein toxins are each coded for by a single gene (monocistronic). According to Federici, (1993) *cited by* Chatterjee et al., (2007), these advantages have favoured the commercialization of *Bt* products internationally.

In addition to strains that kill pests, some strains also kill nematodes. It has been shown that several *Bt* isolates are highly toxic to larvae and adults of *H. contortus*, *Trichostrongylus colubriformis* and *O. circumcincta in vitro*, thereby indicating that these strains of this bacterium may hold some promise as anthelmintics (Kotze et al*.,* 2005). Given that *Bt* is toxic to both larval and adult nematodes (Kotze et al*.,* 2005), it can be utilised as an anthelmintic targeting adult nematodes of the gastrointestinal tract, and/or the free-living larval stages that develop in faeces or on pastures. *B. thuringiensis* would be an ideal biocontrol candidate due to its low mammalian toxicity (Siegel, 2001), and the species specificity shown by particular endotoxin groups (Schnepf et al*.,* 1998). While current chemotherapeutic approaches to animal-parasitic nematode control are aimed almost exclusively at the adult stages, the potential contribution of non-chemical strategies aimed at reducing the levels of larvae developing on pastures has been recognised (Larsen et al., 1998; Larsen 2000).

Kotze et al. (2005) discovered that several uncharacterised *Bt* strains have significant activity against the eggs and larvae of the parasitic nematode *Trichostrongylus colubriformis*. However, OôGrady et al. (2007) found that a significant time constraint exists where *Bt* is fed to livestock to control GIN. A time delay occurs during which *Bt* spores germinate, followed by vegetative cell growth and the development of crystal toxins. However, freshly hatched larvae are more susceptible to *Bt* than later stages, and this has resulted in a significant reduction in the ability of the *Bt* isolates to inhibit larval development.

Mechanism of action of *B. thuringiensis*

The crystal toxin proteins from *B. thuringiensis* are widely used for biological control (Schnepf et al., 1998; Lacey et al., 2001). When conditions for bacterial growth are not optimal, *Bt* forms spores. Spores are the dormant stage of the bacterial life cycle, when the organism waits for better growing conditions. Unlike many other bacteria, when *Bt* develops into spores, it also produces a crystal protein protoxin. This crystal protein is the insect-toxic component of *Bt*. After the crystal is ingested, it dissolves in the alkaline gut, and then the digestive enzymes break down the protoxin into the *Bt* insecticidal components called the delta-endotoxins. The delta-endotoxin binds to the cells lining the midgut membrane, creating pores in the membrane. The haemolymph flows into the gut through the pores, where the gut bacteria proliferate and kill the pest with ñperitonitisò. The spore germinates after the gut membrane is broken; it then reproduces and makes more spores (Swadener, 1994). *Bt* uses the same mechanism to kill nematodes (Wei et al., 2003). However, Kotze et al. (2005) have shown that the toxicity of individual *Cry* proteins vary towards nematodes. Wei et al. (2003) showed that Cry5B, Cry6A, Cry14A and Cry21A are toxic to larvae of a number of free-living nematodes, and that Cry5B, 14A, 21A were also highly toxic to the free-living larval stages of the rodent-parasitic nematode, *Nippostrongylus brasiliensis* (Travvasos). Both studies found that Cry proteins cause damage to the intestine of nematodes as with insects.

Kotze et al. (2005) identified two *Bt* strains that showed activity against three economically important nematode parasites of livestock. This study further extended findings of activity of *Bt* against larval stages of nematodes by showing that *Bt* is also toxic towards the adult stages of nematode parasites. This indicates that the nematode adult life stages, which impact directly on animal health, possess the necessary components (proteases, receptors) for activation of *Bt* isolates, and are susceptible to their toxic effects. The isolation of these biocontrol agents is outlined in Table 1.6.

Table 1.6: Isolation of *D. flagrans, C. rosea,* and *B. thuringiensis*

Biocontrol Agent	**Sources**	**Selective Medium**	**Identification**	**Uses**	**Reference**
D. flagrans	Organic farm soil and compost	Tetracycline Chloride-water agar	Based on morphology of trapping structures and conidia	To trap larval stages 1, 2, and 3.	Ghahfarokhi et al., 2004
C. rosea	Cultivated soils, forests, grassland and woodlands, heathland, freshwater and coastal soil	Potato Dextrose Agar	white colonies, phiallidic conidiophores, and roundish conidia	To degrade nematode cuticle and penetrate the host	Gan et al., 2007
B. thuringiensis	Dung of sheep, cattle, goat, deer, horses, and rabbits; soil from grazing pastures; silage and garden compost.	Nutrient agar	Crystalliferous Spore forming *Bacillus* colonies	To destroy larval and adult nematodes and free-larval stages of parasitic nematodes	Zhang et al., 2000

1.6 References

Ademola, T.O., Fagbemi, B.O., Idowi, S.O. 2005. Anthelmintic activity of extracts of *Spondias mombin* against gastrointestinal nematodes of sheep: Studies in *In vitro* and *In vivo*. Trop. An. Health Prod. 37, 233-235.

Athanasiadou, S., Kyriazakis, I., Jackson, F., Coop, R.L. 2001. Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: *in vitro* and *in vivo* studies. Vet. Parasitol. 99, 199-204.

Barron, G.L. 1977. Observations on predatory fungi. Can. J. Bot. 57, 187-193.

Burke, J.M., Terrill, T.H., Kallu, R.R., Miller, J.E., Mosjidis, J.A. 2007. Use of copper oxide wire particles to control gastrointestinal nematodes in goats. J. An. Sci. 85, 2753-2761.

Chatterjee, S.N., Bhattacharya, T., Danger, T.K., Chandra, G. 2007. Ecology and diversity of *Bacillus thuringiensis* in soil environment. Af. J. Biotech. 6, 1587-1591.

Coles, G.C. 1986. Anthelmintic resistance in sheep. Vet. Clin. North Am. Food An. Pract. 2, 423ï 432.

Coles, G.C., Borgsteede, F.H.M., Geerts, S. 1994. Anthelmintic resistance in nematodes of farm animals. European Commission, Brussels. Pp191.

Conway, D.P. 1964. Variance in effectiveness of thiabendazole against *Haemonchus contortus* in sheep. Am. J. Vet. Res. 25, 844ï 845.

Cooke, R.C. 1969. Two nematode-trapping Hyphomycetes, *Dudddingtonia flagrans* gen. et comb. nov. and *Monacrosporium mutabilis* nov. Trans. Br. Mycol. Soc. 53, 315-319.

Cooke, R.C., Godfrey, B.E.S. 1964. A key to the nematode-destroying fungi. Trans. Br. Mycol. Soc. 47, 61-74.

Corwin, R.M. 1997. Economic gastrointestinal parasitism of cattle. Vet. Parasitol. 72, 451-460.

Cox, J. 1999. The nature conservation of dung. Br. Wildlife 11, 28-36.

Dalton, J.P., Mulcachy, G. 2001. Parasite vaccine-a reality. Vet. Parasitol. 98, 149-167.

De Barjac, H. Franchon, E. 1990. Classification of *Bacillus thuringiensis* strains. J. Entomophoga 35, 233-240.

De, S., Sanyal, P.K. 2009. Biological control of helminth parasites by predatory fungi. Vetscan 4, 1-8.

Desta, B., 1995. Ethiopian traditional herbal drugs. Part I. Studies on the toxicity and therapeutic activity of local taenicidal medications. J. Ethnopharm. 45, 27ï 33.

Dong, J.Y., Zhao, Z.X., Cai, L., Liu, S.Q., Zhang, H.R., Duan, M. 2004. Nematicidal effect of freshwater fungal cultures against the pine-wood nematode *Bursaphelenchus xylophilus*. Fungal Divers, 15, 125ï 135.

Drudge, J.H. Elem, G. 1964. Field studies on parasite control in sheep: comparison of thiabendazole, ruelene, and phenothiazine, Am. J. Vet. Res. 25, 1512ï 1518.

Drudge, J.H., 1957. Strain variation in the response of sheep nematodes to the action of phenothiazine: II. Studies on pure infections of *Haemonchus contortus*. Am. J. Vet. Res. 18**,** 317ï 325.

Entwistle, P.F., Cory, J.S., Bailey, M.J., Higgs, S. (Eds.), 1993. *Bacillus thuringiensis*, an Environmental Biopesticide: Teory and Practice. John Wiley, Chichester.

Faedo, M., Krecek, R.C. 2002. Possible application of a nematophagous fungus as a biological control agent of parasitic nematodes on commercial sheep farms in South Africa. J. S. Af. Vet. Assoc. 73, 31ï 35.

Fontenot, M.E., Miller, J.E., Peña, M.T., Larsen, M., Gillespie, A. 2003. Efficiency of feeding *Duddingtonia flagrans* chlamydospores to grazing ewes on reducing availability of parasitic nematode larvae on pasture. Vet. Parasitol. 118, 203-213.

Gan, Z., Yang, J., Tao, N., Yu, Z., Zhang, K. 2007. Cloning and expression analysis of a chitinase gene *Crchi1* from the mycoparasitic fungus *Clonostachys rosea* (syn. *Gliocladium roseum*). J. of Micro. 45, 422-430.

Gasbarre, L.C., Leighton, E.A., Davies, C.J. 1990. Genetic control of immunity to gastrointestinal nematodes of cattle. Vet. Parasitol. 37, 257ï 272.

Geerts, S., Gryseels, B. 2000. Drug resistance in human helminths: current situation and lessons from livestock. Clin. Micro. Rev. 13, 207-222.

Georgi, J.R. 1969. Parasitology for Veterinarians. W.B. Saunders Company. London.

Ghahfarokhi, M.S., Abyaneh, M.R., Bahadori, S.R., Eslami, A., Zare, R., Ebrahimi, M. 2004. Screening of soil and sheep faecal samples for predacious fungi: Isolation and characterization of the nematode-trapping fungus *Arthrobotrys oligospora.* Iran. Biomed. J. 8, 135-142.

Gibson, T.E. 1960. Some experiences with small daily doses of phenothiazine as a means of control of strongylid worms in the horse. Vet. Rec. 72, 37ï 41.

Githiori, J.B., Höglund, J., Waller, P.J., Baker, R.L. 2002. Anthelmintic activity of preparations derived from *Myrsine africana* and *Rapanea melanophloeos* against the nematode parasite, *Haemonchus contortus*, of sheep. J. Ethnopharm. 80, 187-191.

Grade, J.T., Longok, A. 2002. Karamajong scientists: participatory field trial of a local dewormer. In: Proceedings of the Uganda Veterinary Association Scientific Conference, September 28ï 29, 2000.

Gray, A. 1993. Management of vertisols in Sub-Saharan Africa. Preseedings of a conference held at ILCA, Addis Ababa, Ethiopia. FAO Corporate Document Repository.

Gronvold, J., Wolstrup, P., Larsen, M., Henriksen, S.A., Narsen, P. 1993. Biological control of *Ostertagia ostertagi* by feeding selected nematode-trapping fungi to calves. J. Helminthol. 67, 31-36.

Holmes, P.H. 1987. Pathophysiology of nematode infections. Int. J. Parasitol. 17, 443-451.

Hördegen, P., Hertzberg, H., Heilmann, J., Langhans, W., Maurer, V. 2003. The anthelmintic efficacy of five plant products against gastrointestinal trichostrongylids in artificially infected lambs. Vet. Parasitol. 117, 51- 60.

Hoste, H. 2001. Adaptive physiological processes in the host during gastrointestinal parasitism. Int. Parasitol. 31, 231-244.

Hsu, C., Levine, N.D. 1977. Degree-day concept in development of infective larvae of *Haemonchus contortus* and *Trichostrongylus colubriformis* under constant and cyclic condition. Am. J. Vet. Res. 38, 1115-1119.

Huang, X., Li, Y., Su, Y., Ding, J., Zhang, K. 2009. The influences on conidiophores pleomorphosm in *Clonostachys rosea* and RAPD analysis to the mutant producing only verticillate conidiophores.

Keyyu, J.D., Kyvsgaard, N.C., Monrad, J., Kassuku, A.A. 2005. Epidemiology of gastrointestinal nematodes in cattle on traditional small-scale dairy and large-scale dairy farms in Iringa district Tanzania. Vet. Parasitol. 127, 285-294.

Knox, D.P. 2000. Development of vaccines against gastrointestinal nematodes. Parasitology 120, 53-61.

Knox, M.R., Faedo, M. 2001. Biological control of field infections of nematodes parasites of young sheep with *Duddingtonia flagrans* and effects of spore intake on efficacy. Vet. Parasitol. 101, 155-160.

Kotze, A.C., OôGrady, J., Gough, J.M., Pearson, R., Bagnall, N.H., Kemp, D.H. Akhurst, R.J. 2005. Toxicity of *Bacillus thuringiensis* to parasitic and free-living life-stages of economically important nematode parasites of livestock. Int. J. Parasitol. 35, 1013ï 1022.

Krattiger, F. 1997. Insect resistant crops: A case study of *Bacillus thuringiensis* and its transfer to developing countries. ISAAA briefs, no. 2:42.

Lacey, L.A., Frutos, R., Kaya, H.K., Vale, P. 2001. Insect pathogens as biological control agents: do they have a future? Biol. Control 21, 230ï 248.

Larsen, M. 1999. Biological control of helminths. Int. J. Parasitol. 29, 139-146.

Larsen, M. 2000. Prospects for controlling animal parasitic nematodes by predacious micro fungi. Parasitology 120, 121ï 131.

Larsen, M. 2006. Biological control of nematode parasites in sheep. J. An. Sci. 84, E133-E139.

Larsen, M., Faedo, M., Waller P.J., Hennessy, D.R. 1998. The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: Studies with *Duddingtonia flagrans*. Vet. Parasitol. 76, 121-128.

Larsen, M., Faedo, M., Waller, P.J. 1994. The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: survey for the presence of fungi in fresh faeces of grazing livestock in Australia. Vet. Parasitol. 53, 275ï 281.

Larsen, M., Nansen, P., Gronvold, J., Wolstrup, J., Henriksen, A. 1992. *In vivo* passage of nematophagous fungi selected for biocontrol of parasitic nematodes in ruminants. J. Helminth. 66, 137-141.

Larsen, M., Nansen, P., Wolstrup, J., Gronvold, J., Henriksen, S.A., Zorn, A. 1995. Biological control of trichostrongylus in calves by the fungus *Duddingtonia flagrans* fed to animals under natural grazing conditions. Vet. Parasitol. 60, 321-330.

Larsen, M., Wolsrup, J., Henriken, S.A., Dackman, C., Grovold, J., Nansen, P. 1991. *In vitro* stress selection of nematophagous fungi for biocontrol of parasitic nematodes in ruminants. J. Helminth. 65, 193-200.

Leighton, E.A., Murrell, K.D., Gasbarre, L.C. 1989. Evidence for genetic control of nematode egg-shedding rates in calves. J. Parasitol. 75, 498-504.

Li, G.Q., Huang, H.C., Kokko, E.G., Acharya, S.N. 2002. Ultra structural study of mycoparasitism of *Gliocladium roseum* on *Botrytis cinera*. Bot. Bul. of Aca. Sinica. 43, 211-218.

Li, J., Yang, J.K., Huang, X.W., Zhang, K.Q. 2006. Purification and characterization of an extracellular serine protease from *Clonostachys rosea* and its potential as a pathogenic factor. Process Biochem. 41, 925-929.

Maichomo, M.W., Kagira, J.M., Walker, T. 2004. The point prevailence of gastrointestinal parasites in calves, sheep, and goat in Magadi division, South-West Kenya. Ond. J. Vet. Res. 71, 257-261.

Malan, F.S., Van Wyk, J.A., Wessels, C. 2001. Clinical evaluation of anaemia in sheep: early trials. Onderstepoort J. Vet. Res. 68, 165ï 174.

McKenna, P.B. 1981. The diagnostic value and interpretation of faecal egg count in sheep. N.Z. Vet. J. 29, 129- 132.

McLeon, R.S. 1995. Cost of major parasites to the Australian livestock industry. Int. J. Parasitol. 25, 1363-1367.

Mendoza de Gives, P., Crepso, J.F., Rodriquez, D.H., Prats, V.V., Hernandez, E.L., Fernandez, G.E.O. 1998. Biological control of *Haemonchus contortus* infective larvae in ovine faeces by administering an oral suspension of *Duddingtonia flagrans* chlamydospores to sheep. J. Helminth. 72, 343ï 347.

Merck and Co., Inc. 2008. The Meck Veterinary Manual. USA. http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/191512.htm.

Min, B.R., Hart, S.P. 2003. Tannins for suppression of internal parasites. J. An. Sci. 81, 102ï 109.

Min, B.R., Pomroy, W.E., Hart, S.P., Sahlu, T. 2004. The effect of short-term consumption of a forage containing condensed tannins on gastrointestinal nematode parasite infections in grazing weather goats. Small Rumin. Res. 51, 279ï 283.

Minja, M.M. 1994. Medicinal plants used in the promotion of animal health in Tanzania. Rev. Sci. Tech. 13, 905ï 925.

Mitreva, M.D.S., Zarlenga, J.P., McCoorter, D.P.J. 2007. Parasitic nematodes from genomes to control. Vet. Parasitol. 143, 31-42.

Molan, A.L., Waghorn, G.C., Min, B.R., McNabb, W.C. 2000. The effect of condensed tannins from seven herbages on *Trichostrongylus colubriformis* larval migration *in vitro* Folia Parasitologica 47, 39ï 44.

Morandi, M.A.B., Maya, L.A., Mizubuti, E.S.G., Alfenas, A.C., Barbosa, J.G. 2003. Suppression of *Botrytis cinera* sporulation by *Clonostachys rosea* on rose debris: a valuable component in *Botrytis blight* management in commercial greenhouse. Biol. Control 26, 311ï 317.

Navon, A. 2000. Bioassays of *Bacillus thuringiensis* products used against agricultural pests. In, Bioassay of Entomopathogenic Microbes and Nematodes. (Ed), Navon, A., Ascher, K.R.S. CABI Publishing, New York.

Nienzen, J.H., Charleston, W.A.G., Robertson, H.A., Shelton, D., Waghorn, G.C., Green, R. 2002. The effect of feeding sulla (*Hedysarum coronarium*) or Lucerne (*Medicago sativa*) on lamb parasite burdens and development of immunity to gastrointestinal nematodes. Vet. Parasitol. 105, 229ï 245.

O`Connor, L., W. Stephen and W.W. Brown, 2006. Ecology of the freeliving stages of major Trichostrongyloid parasites of sheep. Vet.Parasitol. 142, 1ï 15

OôGrady, J.O., Akurst, R.J., Kotze, A.C. 2007. The requirement for early exposure of *Haemonchus contortus* larvae to *Bacillus thuringiensis* for effective inhibition of larval development. Vet. Parasitol. 150, 97-103.

Oslon, O.W. 1974. Animal Parasites: Their Life Cycles and Ecology. Dover Publications Inc., New York.

Paolini, V., Dorchies, P., Hoste, H. 2003. Effects of sainfoin hay on gastrointestinal with nematodes in goats. Vet. Rec. 152, 600ï 601.

Peña, M.T., Miller, J.E., Fontenot, M.E., Gillespie, A., Larsen, M., 2002. Evaluation of *Duddingtonia flagrans* in reducing infective larvae of *Haemonchus contortus* in faeces of sheep. Vet. Parasitol. 103, 259ï 265.

Perry, B.D., McDermott, J.J., Randolph, T.F., Sones, K R., Thornton, P.K. 2002. Investing in Animal Health Research to Alleviate Poverty. International Livestock Research Institute, Nairobi, Kenya.

Perry, B.D., Radolph, T.F. 1999. Improving assessment of the economic impact of parasitic diseases in production animals. Vet. Parasitol. 84, 143- 166.

Poynter, D., Hughes, D.L. 1958. Phenothiazine and piperazine, an efficient anthelmintic mixture for horses. Vet. Rec. 70, 183ï 1188.

Prichard, R.K., Roulet, A. 2007. ABC transporters and ß-tubulin in macrocyclic lactone resistance: prospects for marker development. Parasitology 134, 1123-1132.

Quesada-Moraga, E., Garcia-Tovar, E., Vaverde-Garcia, P., Santiago-Alvarenz, C. 2004. Isolation, geographical diversity and insecticidal activity of *Bacillus thuringiensis* from soils in Spain. Microbiol. Res. 159, 59-71.

Sayers, G., Sweeney, T. 2005. Gastrointestinal nematode infection in sheepð a review of the alternatives to anthelmintics in parasite controls. An. Health Res. Rev. 6, 159ï 171.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, Dean, D.R. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Micro. Mol. Biol. Rev. 62, 775ï 806.

Schroers, H.J., Samuels, G.J., Seifert, K.A., Gams, W. 1999. Classification of the mycoparasite *Clonostachys rosea* in *Clonostachys* as *C. rosea*, its relationship to *Bionectria ochroleuca*, and notes on other *Gliocladium*-like fungi. Mycologia 91, 365ï 385.

Senel, S., McClure, S.J. 2004. Potential applications of chitosan in veterinary medicine. Adv. Drug Deliv. Rev. 56, 1467ï 1480.

Shaik, S.A., Terrill, T.H., Miller, J.E., Kouakou, B., Kannan, G., Kallu, R.K. Mosjidis, J.A. 2004. Effects of feeding *Sericea lespedeza* hay to goats infected with *Haemonchus contortus*, S.A. J. An. Sci. 34, 234ï 236.

Shimoda, J., Onishi, H., Machida, Y. 2001. Bioadhesive characteristics of chitosan microspheres to the mucosa of rat small intestine. Drug Dev. Ind. Pharm. 27, 567ï 576.

Siddiqui, Z.A., Mahmood, I. 1996. Biological control of plant parasitic nematodes by fungi: a review. Bior. Tech. 58, 229- 239.

Siegel, J.P. 2001. The mammalian safety of *Bacillus thuringiensis*-based insecticides. J. Invert. Path. 77, 13ï 21.

Skipp, R.A., Chen, L.Y., Yeates, G.W., Glare, R. 2002. Occurrence, morphological characteristics and ribotyping of New Zealand isolates of *Duddingtonia flagrans*, a candidate for biocontrol of animal parasitic nematodes. N.Z. J. Agric. Res. 45, 187ï 196.

Stear, M.J., Doligalska, M., Donskow-Schmelter, K. 2006. Alternatives to anthelmintics for the control of nematodes in livestock. Parasitology 134, 1ï 13.

Stepek, G., Behnke, J.M., Buttle, D.J., Duce, I.R. 2004. Natural plant cysteine proteinases as anthelmintics. Trends Parasitol. 20, 323-327.

Stirling, G.R., Smith, L.J., Licastro, K.A., Eden, L.M. 1998. Development control of root-knot nematode with formulations of the nematode-trapping fungus *Arthrobotrys dactyloides*. Biol. Control 11, 224ï 230.

Stromberg, B.E. 1997. Environmental factors influencing transmission. Vet. Parasitol. 72, 247-264.

Sutton, J.C., Li, D.W. Gang, P. Hai Y., Zhang. P. 1997. *Gliocladium roseum* a versatile adversary of a *Botrytis cinera* in crops. Plant Dis. 81, 316-328.

Swadener, C. 1994. *Bacillus thuringiensis (Bt)*. J. Pest Reform 14, 13-20.

Swartson, H., Nsahlai, I. 2006. Animal Production. MacMillan South Africa Ltd (Pty), Pietermaritzburg.

Tembely, S., Lahlou-Kassie, A., Rege, J.E.O., Sovani, S., Diedhiou, M.L., Baker, R.L. 1997. The epidemiology of nematode infections in sheep in a cool tropical environment. Vet. Parasitol. 70, 129-141.

Terrill, T.H., Kaplan, R.M., Larsen, M., Samples, O.M., Miller, J.E., Gelaye, S. 2001. Anthelmintic resistance on goat farms in Georgia: efficacy of anthelmintics against gastrointestinal nematodes in two selected goat herds. Vet. Parasitol. 97, 261ï 268.

Thamsborg, S.M., Roepstorff, A., Larsen, M. 1999. Integrated and biological control of parasites in organic and conventional production systems. Vet. Parasitol. 84, 169-186.

Tunlid, A., Ahman, J., Oliver, R.P. 1999. Transformation of the nematode-trapping fungus *Arthrobotrys oligospora*, *FEMS.* Micro. Lett. 173, 111ï 116.

Urquhart, G.M., Armour, J., Duncan, J.L., Dunn, A.M., Jennings, F.W. 1987. Veterinary Parasitology. Longman Scientific and Technical. New York, U.S.A.

Valdebenito-Sanhueza, R.M., Sutton, J.C., Perazzolo, I., Czermainski, A.B.C., 1997. Controle biológico de *Botrytis cinera* em morangueiros cultivados em estufa. Fitopatol. Bras. 22, 69ï 73.

Van Wyk, J.A. 1990. Occurrence and dissemination of anthelmintic resistance in South Africa, and management of resistant worm strains. In: Boray, J.C., Martin, P.J., Roush, R.T., (Eds.) Resistance of parasites to antiparasitic drugs. Round Table Conference held at the VIIth International Congress of Parasitology, Paris. MSD. Agvet, Division of Merck, Rahway, New Jersey, 103-113.

Van Wyk, J.A., Stenson, M.O., Van der Merwe, J.S., Vorster, R.J., Viljoen, P.G. 1999. Anthelmintic resistance in South Africa: surveys indicate an extremely serious situation in sheep and goat farming. Ond. J. Vet. Res. 66, 273ï 284.

Vatta, A.F., Letty, B.A., Van der Linde, M.J., Van Wijk, E.F., Hansen, J.W., Krecek, R.C. 2001. Testing for clinical anaemia caused by *Haemonchus* spp. In goats farmed under resource-poor conditions in South Africa using an eye colour chart develop for sheep. Vet. Parasitol. 99, 1-14.

Vercruysse, I., Clarebout, E. 2001. Treatment vs non-treatment of helminth infections in cattle: defining the thresholds. Vet. Parasitol. 98, 195-214.

Waghorn, T.S., Leathwick, D.M., Chen, L.Y., Skipp, R.A. 2003. Efficacy of the nematode-trapping fungus *Duddingtonia flagrans* against three species of gastro-intestinal nematodes in laboratory faecal cultures from sheep and goats. Vet. Parasitol. 118, 227ï 234.157.

Waller, P.J. 1997. Nematode parasite control of livestock in the tropics/subtropics: the need for novel approaches. Int. J. Parasitol. 27, 1193ï 1201.

Waller, P.J., Bernes, G., Thamsborg, S.M., Sukura, A., Richter, S.H., Ingebrigtsen, K., Hoglund, J. 2001. Plants as de-worming agents of livestock in the Nordic countries: historical perspective, popular beliefs and prospects for the future. Acta Vet. Scand. 42, 41-44.

Waller, P.J., Echevarria, F., Eddi, C., Maciel, S., Nari, A., Hansen, J.W. 1996. The prevalence of anthelmintic resistance in nematode parasites of sheep in southern Latin America: general overview. Vet. Parasitol. 62, 181-187.

Waller, P.J., Faedo, M. 1996. The prospects for biological control of the free-living stages of nematode parasites of livestock. Int. J. Parasitol. 26, 915ï 925.

Waller, P.J., Thamsborg, S.M. 2004. Nematode control in ógreenô ruminant production systems. Trends Parasitol. 20, 493ï 497.

Wei, J.Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S.C., Aroian, R.V. 2003. *Bacillus thuringiensis* crystal proteins that target nematodes. Nat. Acad. Sci., USA 100, 2760-2765.

Wu, S.H.W., Papas, A., 1997. Rumen-stable delivery systems. Adv. Drug Deliv. Rev. 28, 323-334.

Yang, X.J., He, Y.X., Chen, F.R., Zheng, L. 2000. Separating and selection of egg-mass fungi of *Meloidogyne* sp. in Fujian province. Fujian J. of Agric. Sci. 15, 12-15.

Zhang, H.Y., Yu, Z.N., Deng, W.X. 2000. Isolation, distribution and toxicity of *Bacillus thuringiensis* from warehouses in China. Crop Prot. 19,449-454.

Zhang, K.Q., Zhao, M.L. 2004. Recent advance of nematophagous fungi from China and with a pecial case on *Clonostachys rosea* (*Gliocladium roseum*). J. of Zhejiang University (Agriculture & Life Sciences). 30, 389-389.

Zhang, L., Yang, J., Nia, Q., Zhao, X., Ye, F., Liang, L., Zhang, K.Q. 2008. Investigation on the infection mechanism of the fungi *Clonostachys rosea* against nematodes using green fluorescent protein. Appl. Microbiol. Biotech. 78, 983-990.

Zhao, M.L., Huang, J.S., Mo, M.H., Zhang, K.Q. 2005. A potential virulence factor involved in fungal pathogenecity serine-like protease activity of nematophagous fungus *Clonostachys rosea*. Fungal Divers. 19, 217-234.

CHAPTER TWO

ISOLATION OF *BACILLUS THURINGIENSIS* (BERLINER) AND *CLONOSTACHYS ROSEA* (SCHROERS) FROM GRAZING PASTURES AND ANIMAL FAECES

M.A. Baloyi, M.D. Laing and K.S. Yobo

Discipline of Plant Pathology, School of Agricultural Sciences and Agribusiness, University of KwaZulu-Natal, Private Bag X01, Scottsville 3201, Pietermaritzburg, South Africa

Abstract

Biological control agents may be an alternative to agrochemicals for the control of livestock nematodes. Strains of *Bacillus thuringiensis* (*Bt*) and *Clonostachys rosea* were isolated from grazing pastures and from faeces collected from pens of livestock. On nutrient agar, *Bt* colonies are circular, white, flat and undulate. *B. thuringiensis* isolates are gram-positive, rod-shaped and form endospores. About 51% of endospore-forming bacteria isolated were *B. thuringiensis*. *C. rosea* were identified by white colonies and the characteristic conidiophores which were branched and showed phialides at the tips. A total of 25 isolates of *B. thuringiensis* and 10 isolates of *C. rosea* were successfully isolated.

2.1 Introduction

Gastrointestinal parasite infection is the most important limiting factor to small ruminant production worldwide, resulting in serious economic losses (Perry et al., 1999; Jackson and Coop, 2000; Perry et al., 2002; Kaplan, 2004). Traditionally, anthelmintics have been used to control these parasites (Borgsteede, 1998). The frequent use of anthelmintics drugs has resulted in resistance, which is currently a major problem in all sheep producing countries, including South Africa (Van Wyk et al., 1999; Vatta et al., 2001). Currently, biological control is considered to be one of the most promising alternatives that might be employed for the control of these parasitic nematodes (Larsen, 1999; Chandrawathani et al., 2003; Waller, 2006; De and Sanyal, 2009). Consequently, the number of studies on how to isolate, screen and characterize biocontrol agents of livestock nematodes is growing.

Some parasporal crystalline inclusions of *Bacillus thuringiensis* (*Bt*) (Berliner) have shown strong activity against both insects and nematodes (Lee and Pankhurst, 1992). The latter was discovered by Waller (2003) and confirmed by Kotze et al*.,* (2005), who reported that several isolates of *Bt* were highly toxic to larvae and adults of livestock nematodes, *Haemonchus contortus* (Rudolphi), *Trichostrongylus colubriformis* (Giles) and *Ostertagia circumcincta* (Stadelmann) in *in vitro* studies. This suggest that *Bt* strains could be used as biological control

agents against adult nematodes in livestock, and the free-living larval stages that develop in pastures, primarily in manure (O'Grady et al., 2007).

The fungus *Clonostachys rosea* (Schroers) has mostly been used as a biological control agent on plant parasitic nematodes. This fungus is commonly found in soil. According to Zhao et al. (2005) and Li et al. (2006), the ability of this fungus to destroy nematodes lies in its ability to penetrate the cuticle, through the production of a germ tube which penetrates the host body and kills it.

Consequently, the aim of this study was to isolate these two biocontrol agents, *Bt* and *C. rosea*, from soil of goat and sheep pens and grazing pastures.

2.2 Materials and Methods

2.2.1 Sample collection

Samples were collected from different sites in the livestock section at the Ukulinga Research Farm, University of KwaZulu-Natal, Pietermaritzburg. The samples comprised of faeces from pens of cattle, sheep and goats, and soil from the grazing pastures of horses, sheep, cattle and goats. Surface soil (15 mm depth) was removed with a spatula and approximately 20g soil and faecal samples were each collected into separate plastic bags. Soil samples were stored in a fridge at 4^0C until used.

2.2.2 Isolation of *Bacillus thuringiensis*

Bacillus thuringiensis was isolated from soil and faecal samples as described by Kotze et al. (2005). Each one gram sample of soil and faecal matter was suspended in 9ml of sterile distilled water and heat treated for 15 min at 80^0C in a rotary water bath. The suspensions were serially diluted, 0.1ml of 10^{-4} dilution was plated onto nutrient agar and kept at room temperature for 4d to sporulate. Spores were harvested into tryptone soy broth (15ml), shaken at 150 rpm for 1wk at 28^0C, then centrifuged (10,000 rpm, 10 min, 4^0C). The pellet was washed once in 0.01M NaCl, and twice in cold sterile distilled water. The final pellet was re-suspended in 4ml of sterile distilled water and stored at -80^0C (as described by Kotze et al., 2005).

2.2.3 Isolation of *Clonostachys rosea*

A basal medium containing $MgS0_4$(0.1g), K_2HPO_4(0.4g), KCl(0.075g), NH_4NO_3(0.5g), glucose (1.5g) and agar power (10g) were mixed and added to 475 ml distilled water and autoclaved. Propamocarb (Previcur) fungicide (0.6g) and crystallized chloramphenicol (0.25g) was added to 50ml of distilled water and separately added to the basal medium.

A one gram sample of soil was suspended in 9ml of sterile distilled water and plated on the medium. The plates were incubated at 25^0C and monitored for the development of lilac colonies. These colonies were subcultured onto Potato Dextose Agar (PDA) and incubated at 25^0C, for 1wk.

2.2.4 Microscopy

To look for the presence of parasporal bodies of *Bt*, an aliquot of a sporulated colony was transferred onto a microscope slide. The slide was heat fixed by passing the slide three times over a Bunsen burner flame, then stained with Coomasie Blue stain (0.133% Coomassie Blue stain in 50% acetic acid), rinsed with distilled water, air-dried and observed under a light microscope (1000x) under oil immersion (Ammons and Ramphersad, 2002).

For *C. rosea*, mycelium was taken from 2wk old cultures grown on PDA and mounted on a slide and stained with lactophenol cotton blue. The specimens were viewed at a 400x magnification.

For electron microscopy, the fungal specimens were fixed overnight in 3% glutaraldehyde in 0.05M cacodylate buffer. The specimens were washed in 0.05M cacodylate buffer twice for 30 min in each wash, then washed in 2% osmium tetroxide for 1 to 4 hours. The specimens were then dehydrated in an ethanol series (30, 50, 70, 80, 90 and 100%) for 10 min each, with 3 changes in the 100% ethanol, with each wash lasting 10min. After dehydration, the specimens were transferred to a critical point drying (CPD) basket submerged in 100% alcohol. The specimen were then placed in a pressure bomb for critical point drying. The specimens were then transferred to scanning electron microscopy (SEM) stubs and examined at 3500x magnification (Kurztman et al., 1974).

2.3 Results

2.3.1 Isolation and identification of *B. thuringiensis*

Details of the different strains of *Bt* isolated from soil are presented in Appendix 1. The colony characteristics of *Bt* isolates were circular, white, flat and undulate (Appendix 1). Out of 548 bacteria isolated, 33 of them were gram-positive and rod-shaped. About 88.14% of the bacterial isolates were gram-negative and 7% were not rod-shaped. Among the 30 Gram-positive isolates, only 25 of them were endospore formers. Thus, 51 % of the bacterial isolates were *Bacillus* spp.

Bacillus thuringiensis was isolated from 15 out of 35 (43%) soil samples. The number of *Bt* isolates found was similar in soil samples from sheep pens and sheep grazing pasture, with each generating 21% of *Bt* isolates. Most *Bt* isolates were found in the soil from goat grazing pasture (25%). The soil from the cattle pen had fewer *Bt* isolates (14.3%), whereas the soil from cattle kraal and horse pasture had no *Bt* (0%). Amongst the 548 bacterial colonies examined, 28 of them were identified as *Bt* isolates, resulting in a mean *Bt* index of 6.1% (Table 2.1).

Table 2.1: Sources and distribution of *Bacillus thuringiensis* isolates from soil

Sample habitat	Number of soil samples examined	Total samples with *Bt*	Number of colonies Examined	Number of *Bt* colonies	*Bt* %
Sheep pen soil	5	3	86	6	6.9
Goat pasture soil	5	3	112	7	6.3
Cattle pen soil	5	2	97	4	4.1
Sheep pastures	5	3	73	5	6.9
Goat pen soil	5	4	90	6	6.7
Cattle kraal soil	5	-	87	-	0
Horse pasture soil	5	-	76	-	0
Total	**35**	**15**	**548**	**28**	**6.1**

Bt % was calculated as the number of *B. thuringiensis* colonies divided by the total number of bacterial colonies examined.

2.3.2 Isolation and identification of *C. rosea*

Colonies of *C. rosea* were velvety in texture. The colours of colonies were initially white, and then became yellow in some isolates. The white colonies had a hyaline septate hyphae and defined branching patterns were observed for the isolates. Conidiophores were straight, with conidia produced at the tip, which were rod-shaped.

Eight isolates of *C. rosea* were isolated from sheep grazing pasture and goat grazing pasture, with six of the isolates being isolated from the goat grazing pasture (Table 2.2).

Table 2.2: Source of *Clonostachys rosea* isolates

Source of soil	**Fungal Isolate**
Sheep grazing pasture	Paec1M
Sheep grazing pasture	Paec2M
Goat grazing pasture	Paec3M
Goat grazing pasture	Paec4M
Goat grazing pasture	Paec5M
Goat grazing pasture	Paec6M
Goat grazing pasture	Paec7M
Goat grazing pasture	Paec8M

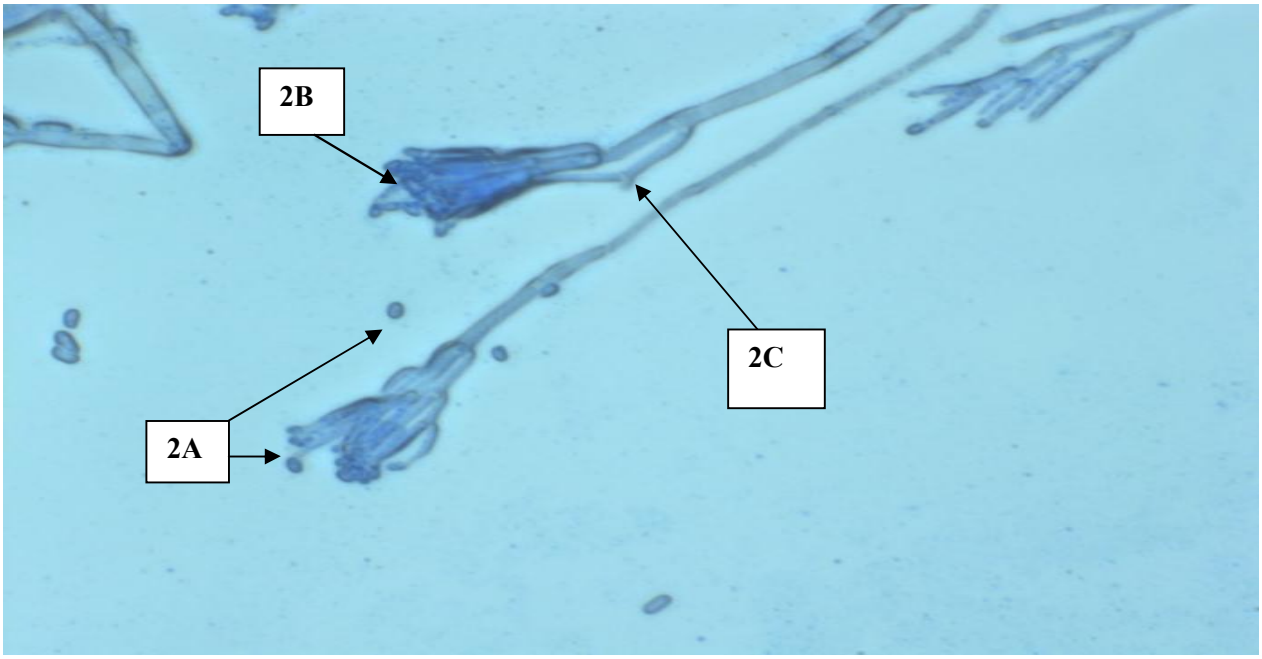

Fig. 2.1: Light microscope image of *C. rosea* (x400) stained with lactophenol cotton blue stain. Fig. 2A shows the conidia produced at the tip of the conidiophores. Fig. 2B shows the branching of the fungus. Fig. 2C shows the septate hyphae.

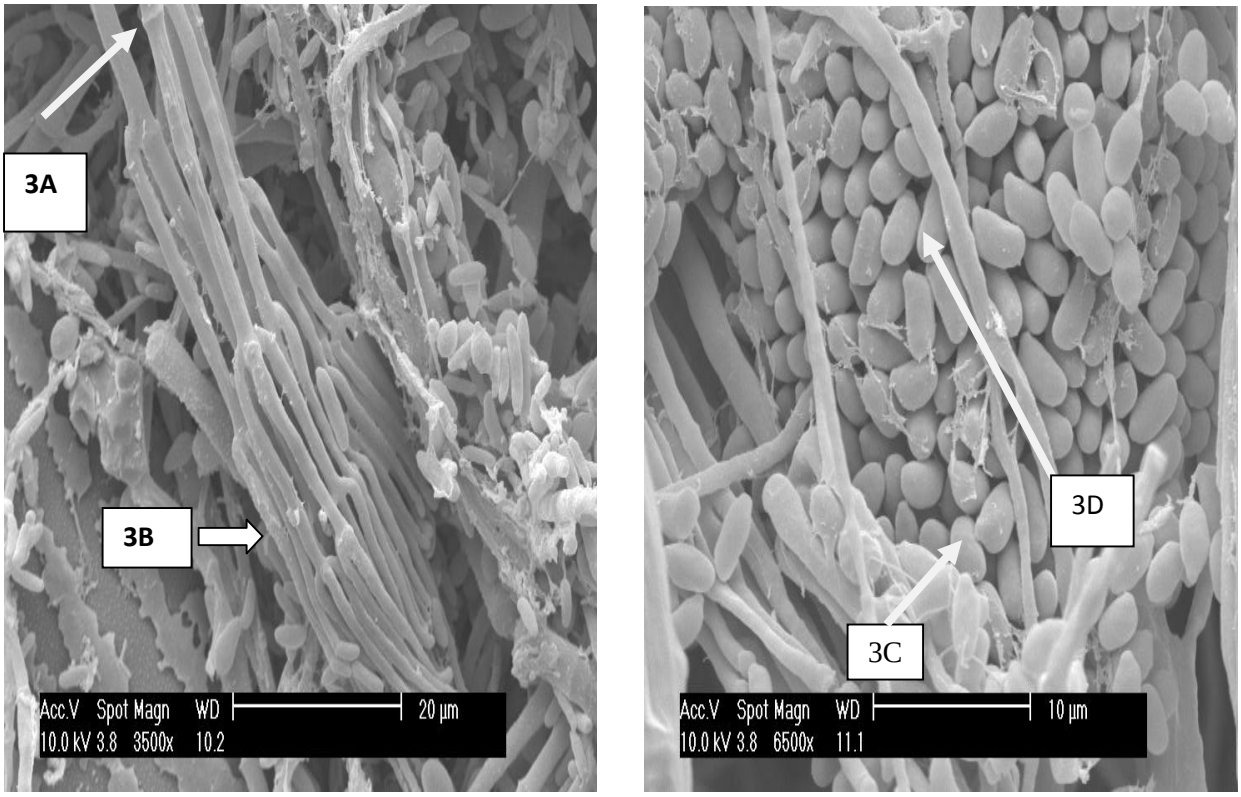

Fig. 2.2: Scanning electron microscopy of colonies of *C. rosea* were velvety in texture. Fig. 3A shows the septate hyphae, Fig. 3B shows the branching patterns of the fungus, Fig. 3C and 3D shows the conidia.

The colonies were whitish in colour. Hyaline septate hyphae and defined branching patterns were observed for the isolates. Conidiophores were straight, with conidia produced at the tip, which were rod-shaped (Fig. 2.1 and 2.2).

2.4 Discussion

This study showed that the soils from the grazing pastures of livestock are rich in *Bt.* This confirms the findings of Martin and Travors, (1989) and Attathom et al. (1995), who reported that *Bt* can be isolated from most soils, including desert, beach and tundra soils. Das et al. (2008) noted that *B. thuringiensis* strains live in diverse habitats, and play an important role in soil ecology, such as maintaining soil structure, nutritional status, degradation of pollutants and the control of several pests. The number of *Bt* isolates depended upon the source of the soil sample,

which might be due to different nutrient availability of the soils, as suggested by Glare and O-Callaghan (2000). Goat pen soil and sheep pen soil gave similar numbers of *Bt* isolates. Soil from cattle grazing pasture produced only four isolates, while there were no *Bt* isolates found from soils of the cattle kraal and horse grazing pasture. The morphological characteristics of the *Bt* isolates are similar to those described by Chatterjee et al. (2007).

The presence of the parasporal bodies was revealed by the presence of numerous dark-blue staining objects. Ammons and Ramphersad, (2002) reported the importance of using stained specimens and light microscopy instead of phase-contrast microscopy, and described dark-blue, rod-shaped staining parasporal bodies such as those observed.

Clonostachys rosea has mostly been isolated from soil (Sutton, 1997). This fungus was present in grazing pasture soils collected from the livestock section at Ukulinga Research Farm. The goat grazing pasture soil produced 63% of the *C. rosea* isolates, more than the sheep and the cattle grazing pastures. All of these isolates had oval shaped conidia with septate hyphae. The characteristic of the colony development was as described by Schroers (1997), Gan et al. (2007) and St. German, (1996). Hyaline septate hyphae with defined branching patterns resembling that of *C. rosea* were observed.

2.5 References

Ammons, D., Ramphersad, J. 2002. Usefulness of staining of the parasporal bodies when screening for *Bacillus thuringiensis*. J. Invert. Parasitol. 79, 203-204.

Attathom, T., Chongrattanameteekul, W., Chanpaisang, J., Sirijan, R. 1995. Morphological diversity and toxicity of delta-endotoxin produced by various strains of *Bacillus thuringiensis.* Bull. Ento. Res. 85, 167-173.

Borgsteede, F.H.M. 1998. Gastrointestinal helminthiasis: Anthelmintic resistance and how to prevent and control. Symp. Parasitol. Int. 47, 23-48.

Chandrawathani, P., Jamnah, O., Waller, P.J, Larsen, M., Gillespie, A.T., Zahari, W.M. 2003. Biological control of nematode parasites of small ruminants in Malaysia using the nematophagous fungus *Duddingtonia flagrans.* Vet. Parasitol. 117, 173-183.

Chatterjee, S.N., Bhattacharya, T., Danger, T.K., Chandra, G. 2007. Ecology and diversity of *Bacillus thuringiensis* in soil environment. Af. J. Biotech. 6, 1587-1591.

Das, J., Das, B., Dangar, T.K. 2008. Microbial populations and *Bacillus thuringiensis* diversity in saline rice field soils of coastal Orissa, India. Af. J. Micro. Res. 2, 326-331.

De, S., Sanyal, P.K. 2009. Biological control of helminth parasites by predatory fungi. Vetscan 4, 1-8.

Glare, T.R., O-Callaghan, M. 2000. *Bacillus thuringiensis*: Biology, Ecology and Safety. Wiley, Chichester.

Gan, Z., Yang, J., Tao, N., Yu, Z., Zhang, K. 2007. Cloning and Expression Analysis of a Chitinase Gene *Crchi1* from the Mycoparasitic Fungus *Clonostachys rosea* (syn. *Gliocladium roseum*). The J. of Micro. 45, 422-430.

Jackson, F., Coop, R.L. 2000. The development of anthelmintic resistance in sheep nematodes. Parasitology 120, 95ï 107.

Kaplan, R.M. 2004. Drug resistance in nematodes of veterinary importance: A status report. Trends Parasitol. 20, 477-481.

Kotze, A.C., OôGrady, J., Gough, J.M., Pearson, R., Bagnall, D.H., Akhurst, R.J. 2005. Toxicity of *Bacillus thuringiensis* to parasitic and free-living life stages of nematode parasitic of livestock. Int. Parasitol. 35, 1013-1022.

Kurtzman, C.P., Baker, F.L. Smiley, M.J. 1974. Specimen holder to critical point dry microorganisms for scanning electron microscopy. Appl. Micro. 28, 708-712.

Larsen, M. 1999. Biological control of helminths. Int. J. Parasitol. 29, 139-146.

Lee, K.E., Pankhurst, C.E. 1992. Soil organisms and sustainable productivity. Austr. J. Soil Res. 30, 855-892.

Li, J., Yang, J.K., Huang, X.W., Zhang, K.Q. 2006. Purification and characterization of an extracellular serine protease from *Clonostachys rosea* and its potential as a pathogenic factor. Process Biochem. 41, 925-929.

Martin, P.A.W., Travers, R.S. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Env. Micro. 55, 2437-2442.

OôGrady, J., Akhurst, R.J., Kotze, A.C. 2007. The requirement for early exposure of *Haemonchus contortus* larvae to *Bacillus thuringiensis* for effective inhibition of larval development. Vet. Parasitol. 150, 97-103.

Perry, B.D., Randolph, T.F. 1999. Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. Vet. Parasitol. 84, 145ï 168.

Perry, B.D., Randolph, T.F., McDermott, J.J., Sones, K.R., Thornton, P.K. 2002. Investing in Animal Health Research to Alleviate Poverty. International Livestock Research Institute (ILRI), Nairobi, Kenya, p. 148.

Schroers, H.J., Samuels, G.J., Seifert, K.A., Gams, W. 1999. Classification of the mycoparasite *Clonostachys rosea* in *Clonostachys* as *C. rosea*, its relationship to *Bionectria ochroleuca*, and notes on other *Gliocladium*-like fungi. Mycologia 91, 365ï 385.

St. Germain, G., Summerbell, R. 1996. Identifying Filamentous Fungi ï A Clinical Laboratory Handbook, 1st ed. Star Publishing Company. Carlifornia, U.S.A.

Sutton, J.C., Li, D.W. Gang, P. Hai Y., Zhang. P. 1997. *Gliocladium roseum* a versatile adversary of a *Botrytis cinera* in crops. Plant Dis. 81, 316-328.

Van Wyk, J.A., Stenson, M.O., Van der Merwe, J.S., Vorster, R.J., Viljoen, P.G. 1999. Anthelmintic resistance in South Africa: surveys indicate an extremely serious situation in sheep and goat farming. Ond. J. Vet. Res. 66, 273-284.

Vatta, A.F., Letty, B.A., Van der Linde, M.J., Van Wijk, E.F., Hansen, J.W., Krecek, R.C. 2001. Testing for clinical anaemia caused by *Haemonchus* spp. in goats farmed under resource ï poor conditions in South Africa using an eye colour chart developed for sheep. Vet. Parasitol. 99, 1-9.

Waller, P.J. 2003. Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy, with emphasis on biological control. An. Health Res. Rev. 4, 35-43.

Waller, P.J. 2006. Sustainable nematode parasite control strategies for ruminant livestock by grazing management and biological control. An. Feed Sci. 126, 277-289.

Zhao, M.L., Huang, J.S., Mo, M.H., Zhang, K.Q. 2005. A potential virulence factor involved in fungal pathogenicity serine-like protease activity of nematophagous fungus *Clonostachys rosea*. Fungal Divers. 19, 217-234.

CHAPTER THREE

IN VITRO SCREENING OF *BACILLUS THURINGIENSIS* (BERLINER) AND *CLONOSTACHYS ROSEA* (SCHROERS) AGAINST LIVESTOCK PARASITIC NEMATODES

M.A. Baloyi, M.D. Laing and K.S. Yobo

Discipline of Plant Pathology, School of Agricultural Sciences and Agribusiness, University of KwaZulu-Natal, Private Bag X01, Scottsville 3201, Pietermaritzburg, South Africa

Abstract

In vitro studies were conducted to determine the efficacy of *Bacillus thuringiensis* (*Bt*) and *Clonostachys rosea* isolates against livestock nematodes in sheep faeces. Suspensions of *B. thuringiensis* spores and *C. rosea* conidia were tested on sheep nematodes in faeces and in water in the laboratory. Following incubation at 25^0C for 7d, treated faecal samples were Baermanized overnight and larvae counted. Twenty-five isolates of *Bt* and 10 of *C. rosea* reduced nematode counts considerably. Isolates of *Bt* and *C. rosea* reduced nematode counts by 28.5% to 62% and 44% to 69.9%, respectively, in the faecal bioassay. In the water bioassay, nematode count reductions of 62% to 85% and 62.7% to 89.3% by *Bt* and *C. rosea*, respectively, were observed. Most of the best nematode-killing isolates were from a goat grazing pasture.

3.1 Introduction

A number of microorganisms have been found to kill nematodes. This includes fungi, bacteria, viruses, insects, mites and some invertebrates (Stirling, 1991; Siddiqui et al., 1996). Sheep farming enterprises in South Africa maintain a flock of approximately 29.2 million sheep (Faedo and Krecek, 2002). The industry faces serious production constraints due to parasitic gastrointestinal nematodes (GIN). These nematodes are known to significantly limit livestock productivity for both commercial and small scale farmers. Parasitism by these nematodes may have direct effects, resulting in acute illness and death, forcing premature slaughter, and rejection of parts of the carcass during meat inspection at abattoirs. They also cause indirect losses due to sub-clinical levels of parasitism (Larsen, 2002). For years, chemical control has been the sole solution applied to the problem. However, the widespread development of anthelmintic resistance (Waller, 2003; Kaplan, 2004; Waller 2006) has made the search for alternative control measures, such as biological control, of utmost importance to the global livestock industry.

Bacillus thuringiensis (*Bt*) (Berliner) is a heterogeneous species of rod-shaped, gram-positive bacterium, which produces *Cry* toxin proteins during sporulation. These are used in insect pest control in agriculture (Schnepf, 1998). However, some *Bt* strains also show activity against

livestock nematodes (Kotze et al., 2005). Kotze et al. (2005) also demonstrated that isolates of this bacterium were able to consistently reduce larval counts and adult populations of these nematodes. Kotze et al. (2005) also demonstrated the capability of *Bt* isolates to kill free-living stages of parasitic nematodes *in vitro.* This toxicity was believed to be due to one or more parasporal inclusions produced during sporulation. The use of *Bt* in control strategies may be better than the traditional use of chemical approaches because they are host specific and do not leave chemical residues. The target species specificity of particular *Cry* proteins prevents off-target effects on beneficial arthropods, and has a low mammalian toxicity (Schnepf, et al., 1998; Siegel, 2001). Lee and Pakhurst (1992) first discovered *B. thuringiensis* in the faeces of herbivorous animals in Japan. Several authors have long found soil to be a natural habitat of *Bt* (Ohba and Aizawa, 1986; Martin and Travers, 1989; Hastowo et al., 1992).

Clonostachys rosea (Schroers) is a soil saprophytic fungus that has been isolated mostly from the soil. This fungus has shown to be parasitic to plant nematodes and other fungi (Zhao et al., 2005; Li et al., 2006). Many researchers have shown the potential of another nematophagous fungus, *Duddingtonia flagrans* (Cooke), to reduce livestock nematode populations in both laboratory and pasture trials (Waller et al., 2001; Peña et al., 2002; Terrill et al., 2004).

The aim of this study was to evaluate the pathogenicity of *Bt* and *C. rosea* isolates on the larvae of a mixed culture of sheep nematodes in *in vitro* trials, conducted in both sheep faeces and in water.

3.2 Materials and Methods

3.2.1 Isolation of biocontrol agents

Pathogenic strains of *Bt* and *C. rosea* were isolated from pasture soils of the livestock section of Ukulinga Research Farm at University of KwaZulu-Natal, Pietermaritzburg, as described in Chapter 2.

3.2.2 Pathogenicity bioassays

3.2.2.1 Preparation of biocontrol agents

Spores of *B. thuringiensis*

Spores of *Bt* were obtained by scraping a frozen culture that was stored at -80^{0}C. These were streaked onto Tryptone Soy Agar (TSA) plates and allowed to grow for 4 d at room temperature. The spores were harvested into 250ml Erlenmeyer flask containing 50 ml of Tryptone Soy Broth. The spores were grown by shaking on an orbital shaker at 150 rpm at 28^{0}C for 2 wks. After incubation, the cultures were centrifuged (10 000 rpm at 4^{0}C for 15 min), washed once in 0.1 mole (M) NaCl and twice in sterile distilled cold water (as described by Kotze et al., 2005). The pellets were suspended in sterile distilled water (40 ml) and the number of spores was determined by a dilution plating technique and adjusted to a concentration of 10^8 spores ml^{-1}.

Conidia of *C. rosea*

Conidia were produced by growing the fungal cultures on 2% water agar for 3 wk in petri dishes. Conidia were recovered by washing the surface of the agar covered with mycelia, with sterile distilled water into a beaker. The conidial suspension was filtered through a double layer of cheese cloth into a sterilized beaker to remove mycelia. The concentration of spores was determined by a haemocytometer and adjusted to a concentration of 10^6 conidia ml^{-1}.

3.2.2.2 Bioassay in sheep faeces

To conduct the bioassay, a modified method of Ghahfarokhi et al. (2004) was used. Faecal samples were collected directly from the rectum of naturally infested sheep that fed on grass pastures. The sheep had a mixed infection of *Haemochus contortus* and *Trichostrogylus* spp. nematodes. The faecal pellets were crushed and mixed thoroughly to homogenize the sample, and then a subsample of 2 g of the faeces was placed in each well of a 24 multiwell microtitre plate, then treated with a *Bt* cell suspension or a *C. rosea* conidial suspension, at a ratio of 1:1 (w/v). The plates were covered with parafilm to prevent evaporation. Small holes were punched in the parafilm above each well to allow in air. Plates were incubated at 25^0C for 7 d and nematode larvae were recovered using the Baermann technique (Ichhpujani and Bhatia, 1998).

3.2.2.3 Bioassay in water

Rearing of nematodes

Faeces were collected directly from the rectum of sheep infected with a mixed culture of nematodes. The faeces were incubated for 4 d at 25^0C. After incubation, the nematodes were extracted using the Baermann technique. Approximately 20 ml of sediment containing larvae was drawn off into McCartney bottles and left to settle at 4^0C for a minimum of 8 h. The nematodes were surface sterilized in 0.525% sodium hypochlorite (v/v) for 5 min, then washed a further three times in sterile distilled water through a 25 µm sieve (Fischer-Le Saux et al., 1998). The number of larvae present in each 1 ml sample was adjusted to 55-65 L2 larvae. One milliliter containing nematodes was poured into a sterilized test tube and 667 µl of biocontrol agent suspension was added, with the Control receiving 667 µl of sterile distilled water. Luria-Bertani (LB) broth (10 µl) was added to the tubes (as described by OõGrady et al., 2007) and the samples were incubated at 25^0C. After 7 d incubation, the numbers of nematode larvae were counted under a dissecting microscope. The living nematodes formed a C-shape while the dead nematodes were just straight.

3.2.3 Statistical analysis

Treatments were arranged in a Randomized Complete Block Design with three replications. The experiment was repeated three times for the faecal culture bioassay and two times for the water

bioassay. The nematode count values were pooled. The nematode counts of the faeces and water bioassays were angular transformed to normalize the data. Data was analysed using ANOVA Genstat® Release 11.1. If the F-test was significant, then the means of nematode counts were separated using Duncanôs Multiple Range Test for *Bt* isolates in the faecal bioassay or Fisherôs least significant difference test (LSD) for *C. rosea* in both water and faecal bioassay and *Bt* in the water bioassay.

The following formula was used to determine the degree of control of nematodes by the isolates tested in faeces:

$$\text{Percentage reduction} = \frac{[C_1 - C_2]}{C_1} \times 100$$

Where, C_1 = mean number of larvae in untreated manure;

C_2 = number of larvae in treated manure

Henderson-Tilton's formula (Henderson and Tilton, 1955) was used to determine the mortality (%) in water bioassay:

$$\text{Corrected mortality (\%)} = \left(1 - \frac{\text{n in Ctl before treatment} \times \text{n in T after treatment}}{\text{n in Ctl after treatment} \times \text{n in T before treatment}}\right) \times 100$$

Where n = number of nematodes, Ctl = control, T = treatment

3.3 Results

3.3.1 Pathogenicity of *B. thuringiensis* and *C. rosea* in faecal samples

The *Bt* isolates were significantly different from each other in their activity against livestock nematodes. Similarly, isolates of *C. rosea* were significantly different in their ability to reduce the number of infective larvae ($P < 0.001$).

Isolates of *Bt* caused nematode mortalities that ranged from 28.5% to 64%. Fourteen isolates caused a reduction of nematode counts of more than 50% (Table 3.1). Three isolates, Bt2(4)M, Bt10(1)M, Bt10(2)M, caused a nematode mortality of more than 60% in faeces. Eleven *Bt*

isolates caused mortality of less than 50%. Isolate Bt7(2)M resulted in the least control of nematodes (28.5%), whereas Bt2(4)M was the most effective *Bt* isolate. Eleven of the isolates (66%) caused nematode mortality in the range of 50% to 58%, with four isolates providing control levels greater than 60% (Table 3.2).

Some isolates of *C. rosea* were effective in reducing nematode populations in faeces by more than 60% (Table 3.1). The best isolate (Paec 8) caused a mortality of 69.9% (Table 3.3). Eight of the 10 *C. rosea* isolates killed more than 50% of the nematodes in faeces. Isolates Paec1, Paec3 and Paec8 were the best three isolates, causing mortalities of 67.9%, 68.3% and 69.9%, respectively (Table 3.3).

Table 3.1: Habitat of isolates of *Bacillus thuringiensis* and *Clonostachys rosea*

		Control levels			
Genus	**Habitat**	**0-49%**	**50-59%**	**60-70%**	**Total number of isolates**
B. thuringiensis	Sheep pen	Bt7(2)M, Bt8(3)M,	Bt11(1)M,		3
	Goat grazing pasture	Bt10(5), Bt13M,	Bt15M, Bt12M, Bt10(4)M,	Bt2(4)M	6
	Cattle pen	Bt1(4)M, Bt5(2)M, Bt1(1)M,	Bt14M	Bt10(2)M	5
	Sheep grazing pasture	Bt3(4)M, Bt10(3)	, Bt9(4)M,Bt9(2)M	Bt10(1)M	5
	Goat pen	Bt4(2)M, Bt5(3)M,	,Bt6(3)M,Bt1(2)M Bt4(1)M, Bt6(2)M		6
C. rosea	Goat grazing pasture	Paec7M		Paec4M, Paec5M, Paec6M Paec8M,	5
	Sheep grazing pasture			Paec1M, Paec2M, Paec3M,	3
	Unknown	B-40	MM		2
TOTAL		**13**	**12**	**10**	**35**

Table 3.2: Mean mortality (%) of gastrointestinal nematodes of sheep that reached L3 after 7 d, following exposure to treatment with *Bacillus thuringiensis* at a concentration of 10^8 spores ml^{-1} in a sheep faeces bioassay

Bacterial isolates	**Mean mortality (%)**	**Transformed means**
Bt 1(1)M	47.0	43.3**cdefg**
Bt 1(2)M	55.2	48.0**efg**
Bt 1(4)M	41.0	39.7**bcd**
Bt 10(2)M	60.5	51.1**fg**
Bt 10(3)M	49.5	44.7**defg**
Bt 10(4)M	54.8	47.9**efg**
Bt 10(5)M	44.0	41.5**bcdef**
Bt 11(1)M	55.2	48.1**efg**
Bt 12M	57.0	49.1**efg**
Bt 13M	45.8	42.4**bcdefg**
Bt 14M	53.0	46.6**efg**
Bt 15M	52.0	46.2**efg**
Bt 2(4)M	64.0	53.2**g**
Bt 3(4)	31.0	33.5**bc**
Bt 4(1)M	56.0	48.5**efg**
Bt 4(2)M	43.0	41.0**bcde**
Bt 5(2)M	42.0	40.4**bcde**
Bt 5(3)M	49.5	44.7**defg**
Bt 6(2)M	58.0	49.6**efg**
Bt 6(3)M	50.2	45.2**efg**
Bt 7(2)M	28.5	33.2**bc**
Bt 8(3)M	45.2	42.2**bcdefg**
Bt 9(2)M	56.2	48.7**efg**
Bt 9(4)M	50.8	45.4**efg**
Bt10(1)M	62.5	52.3**fg**
Control	0.0	00.0**a**
F-ratio		11.65
P-value		< 0.001***
LSD		8.405
CV%		13.8

Means followed by the same letter are not significantly different at P > 0.05
Means were compared using Duncanôs Multiple Range Test at a 5% level
* Denotes significantly different at 5% probability

Table 3.3: Mean mortality (%) of gastrointestinal nematodes of sheep after 7 d, following exposure to treatment with *Clonostachys rosea* isolates at a concentration of 10^6 conidia ml^{-1} in a sheep faeces bioassay

Fungal isolates	Mean Mortality (%)	Transformed mean
Paec8	69.9	56.9**c**
Paec3	68.3	55.8**c**
Paec1	67.9	55.5**c**
Paec4	65.5	54.1**bc**
Paec2	65.0	53.8**bc**
Paec5	60.5	53.3**bc**
Paec6	61.6	51.8**bc**
MM	51.5	45.9**bc**
B-40	48.9	44.5**bc**
P7	46.3	42.2**b**
Control	0.0	0.0**a**
F-ratio		13.03
P-value		< 0.001***
LSD		12.99
CV%		19.3

Means followed by the same letter in the same column are not significantly different at P > 0.05
F-ratio and P-value after angular transformation
* Denotes significantly different at 5% probability

3.3.2 Pathogenicity of isolates in a water bioassay

The best three isolates of *Bt* significantly reduced nematode numbers (P < 0.001). However, there was no difference between the isolates in their ability to kill nematodes *in vitro* in the water bioassay, causing mortality levels of 62.4%, 62.0% and 85.0% (Isolates Bt2(4)M, Bt10M and Bt12M, respectively) (Table 3.4).

The best three *C. rosea* isolates significantly reduced nematode counts in the Water bioassay (P < 0.001). Mortality levels were higher than in the faeces trial. The fungal isolates, Paec1, Paec3 and Paec8, reduced nematode levels by 62.7%, 82.0% and 89.3%, respectively (Table 3.5).

Table 3.4: Mortality (%) of nematodes larvae treated with a *Bacillus thuringiensis* spore suspensions at a concentration of 10^8 cells ml^{-1} for 7 d at 25^0C in a water bioassay

Isolate	Transformed mortality (%)	Mean mortality (%)of nematodes
B2(4)M	0.0850**b**	62.4
B10(1)M	0.0917**b**	62.0
B12M	0.0937**b**	85.0
Ctrl	0.0**a**	0
F-ratio	181.96	
P-value	<0.001	
LSD	0.012	
CV%	8.6	

Means were square-root arcsine transformed
F- ratio and P-values after square root-arcsine transformation
* Denotes significantly different at P Ò0.05

Table 3.5: Mortality of nematodes larvae treated with *Clonostachys rosea* conidial suspensions at a concentration of 10^6 conidia ml^{-1} for 7d at 25^0C in a water bioassay

Isolate	Mean mortality (%) of nematodes
Paec8	89.3**c**
Paec3	82.0**bc**
Paec1	62.7**b**
Ctrl	0.0**a**
F-ratio	41.10
P-value	<0.001***
LSD	21.8
CV%	18.7

Means followed by the same letter are not significantly different P > 0.05

Values are means of three replicate experiment repeated two times

* Denotes significantly different at P Ò0.05

3.4 Discussion

For many years, GIN management has needed an alternative to chemotherapy (Waller et al., 2004), due to drug resistance to anthelmintics and other associated problems caused by chemicals (Sangster, 1999; Jackson and Coop, 2000; Kaplan, 2004; Coles, 2005).

This study evaluated the effect of 25 *Bt* and 10 *C. rosea* isolates for the control of GIN. Isolates of *Bt* and *C. rosea* significantly reduced the number of nematodes in both water and faecal trials, when applied at a concentration of 10^8 and 10^6 spores or conidia ml^{-1} for *Bt* and *C. rosea,* respectively. The results obtained with *Bt* isolates confirmed the work of Waller (2003), Wei (2003) and Kotze et al. (2005), all of whom showed that some *Bt* isolates are capable of killing animal nematodes.

Clonostachys rosea (Schroers) has been used as a biological control agent (BCA) to control plant parasitic nematode. The fungus was also reported to parasitize *Botrytis cinera* fungus (Sutton et al., 1997; Zhao et al., 2005; Li et al., 2006). It has now been shown that it can also attack the larvae of animal nematodes.

Soil samples from sheep pens and a sheep pasture had the lowest numbers of nematicidal isolates for both *Bt* and *C. rosea*, whereas soil from a goat grazing pasture and goat faeces gave the highest number of nematicidal isolates. The best isolates of both *Bt* and *C. rosea* were from a goat grazing pasture, possibly reflecting a low nematode population carried by the flock of goats.

No isolate(s) of either *Bt* or *C. rosea* provided a 100% nematode control. This confirms Larsenôs (2000) finding, that no biological control agent eliminated all infective larval stages entirely, but that they could reduce pasture contamination to such a low level that animals could develop a natural immune response to GIN. The best *C. rosea* isolates were marginally more effective against nematodes than the best *Bt* isolates.

Most isolates of the two biocontrol agents killed nematodes in both the faecal bioassay and in the water bioassay. Sheep are mostly infected with a mixed culture of nematodes, thus an effective biocontrol agent should be able to work against a variety of nematode species (Waghorn et al., 2003). In this study, the faeces were collected from sheep naturally infected with a mixed culture of nematodes. Fortunately, the two groups of biocontrol agents used in these bioassays both reduced the total numbers of the mixed populations of nematodes. Most of the successful BCA research on livestock nematodes has centred on the nematophagous fungus *D. flagrans* (Larsen et al., 1994; Larsen et al., 1998; Waller et al., 2001; Paraud et al., 2006). However, *Bt* and *C. rosea* are competitive BCAs that can be used to reduce the population of livestock nematodes in pastures.

In conclusion, the study showed that locally isolated strains of *Bt* and *C. rosea* had nematicidal effects and could be incorporated in a GIN control programme, as an alternative to chemical anthelmintics. Further studies need to be undertaken to determine the most effective way of administering these BCAs via animal feeds.

3.5 References

Coles, G.C. 2005. Anthelmintic resistance-looking to the future: a UK perspective. Res. Vet. Sci. 78, 99ï 108.

Faedo, M., Krecek, R.C. 2002. Possible application of a nematophagous fungus as a biological control agent of parasitic nematodes on commercial sheep farms in South Africa. J. S.A. Vet. Assoc. 73, 31-35.

Fischer-Le Saux, M., Mauleon, H., Constant, P., Brunel, B., Boermare, N. 1998. PCR-ribotyping of *Xenorhabdus* and *Photorhabdus* isolates from the Caribbean region in relation to the taxonomy and geographic distribution of their nematode hosts. Appl. Env. Micro. 64, 4246ï 4254.

Ghahfarokhi, M.S., Abyaneh, M.R., Bahadori, S.R., Eslami, A., Zare, R., Ebrahimi, M. 2004. Screening of soil and sheep faecal samples for predacious fungi: Isolation and characterization of the nematode-trapping fungus *Arthrobotrys oligospora.* Iran. Biomed. J. 8, 135-142.

Hastowo, S., Lay, B.W., Ohba, M. 1992. Naturally occuring *Bacillus thuringiensis* in Indonesia. J. Appl. Bact. 73, 108-113.

Henderson, C.F., Tilton, E.W. 1955. Tests with acaricides against the brown wheat mite. J. Econ. Ento. 48, 157-161.

Ichhpujani, R.L., Bhatia, R. 1998. Medical Parasitology, Second Ed. Jaypee Brothers Medical Publishers, Ltd., New Delhi, India.

Jackson, F., Coop, R.L. 2000. The development of anthelmintic resistance in sheep nematodes. Parasitology 120, S95ï S107.

Kaplan, M. 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477-481.

Kotze, A.C., O'OðGrady, J., Gough, J.M., Pearson, R., Bagnall, D.H., Akhurst, R.J. 2005. Toxicity of *Bacillus thuringiensis* to parasitic and free-living life stages of nematode parasitic of livestock. Int. Parasitol. 35, 1013-1022.

Larsen, M. 2000. Prospects for controlling animal parasitic nematodes by predacious microfungi. Parasitology 120, 121-131.

Larsen, M. 2002. Biological control in a global perspective- A review with emphasis on *Duddingtonia flagrans*. In, Biological Control of Nematode Parasite Control of Small Ruminants. FAO, Malaysia, Asia. pp. 19-37.

Larsen, M., Faedo, M., Waller, P.J. 1994. The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: survey for the presence of fungi in fresh faeces of grazing livestock. Austr. J. Vet. Parasitol 53, 275-281.

Larsen, M., Faedo, M., Waller, P.J., Hennessy, D.R. 1998. The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: Studies with *Duddingtonia flagrans*. Vet. Parasitol. 76, 121-128.

Lee, K.E., Pankhurst, C.E. 1992. Soil organisms and sustainable productivity. Austr. J. Soil Res. 30, 855-892.

Li, J., Yang, J.K., Huang, X.W., Zhang, K.Q. 2006. Purification and characterization of an extracellular serine protease from *Clonostachys rosea* and its potential as a pathogenic factor. Process Biochem. 41, 925-929.

Martin, P.A.W., Travers, R.S. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Env. Micro. 55, 2437-2442.

OôGrady, J., Akhurst, R.J., Kotze, A.C. 2007. The requirement for early exposure of *Haemonchus contortus* larvae to *Bacillus thuringiensis* for effective inhibition of larval development. Vet. Parasitol. 150, 97-103.

Ohba, M., Aizawa, K. 1986. Distribution of *Bacillus thuringiensis* in soils of Japan. J. Invert. Path. 47, 277-282.

Paraud, C., Pors, I., Chicard, C., Chartier, C. 2006. Comparative efficacy of the nematode-trapping fungus *Duddingtonia flagrans* against *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* in goat faeces: influence of the duration and of the temperature of coproculture. Parasitol. Res. 98, 207-213.

Peña, M.T., Miller, J.E., Fontenot, M.E., Gillespie, A., Larsen, M. 2002. Evaluation of *Duddingtonia flagrans* in reducing infective larvae of *Haemonchus contortus* in faeces of sheep. Vet. Parasitol. 103, 259ï 265.

Sangster, N.C. 1999. Anthelmintic resistance: past, present and future. Int. J. Parasitol. 29, 115ï 124.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Micro. Mol. Biol. Rev. 62, 775-806.

Siddiqui, Z.A., Mahmood, I. 1996. Biological control of plant parasitic nematodes by fungi: a review. Bior. Technol. 58, 229-239.

Siegel, J.P. 2001. The mammalian safety of *Bacillus thuringiensis*-based insecticides. J. Invert. Path. 77, 13-21.

Stirling, G.R. 1991. Biological Control of Plant Parasitic Nematodes: Progress, Problems and Prospects. CAB International, Wallingford, UK.

Sutton, J.C., Li, D.W., Gang, P., Hai, Y., Zhang, P. 1997. *Gliocladium roseum* a versatile adversary of a *Botrytis cinera* in crops. Plant Dis. 81, 316-328.

Terrill, T.H., Larsen, M., Samples, O., Husted, S., Miller, J.E., Kaplan, R.M., Gelaye, S. 2004. Capability of the nematode-trapping fungus *Duddingtonia flagrans* to reduce infective larvae of gastrointestinal nematodes in goat faeces in the southeastern United States: dose titration and dose time interval studies. Vet. Parasitol. 120, 285ï 296.

Waghorn, T.S., Leathwick, D.M., Chen, L.Y., Skipp, R.A. 2003. Efficacy of the nematode-trapping fungus *Duddingtonia flagrans* against three species of gastrointestinal nematodes in laboratory faecal cultures from sheep and goats. Vet. Parasitol. 118, 227-234.

Waller, P.J. 2003. Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy with emphasis on biological control. An. Health Res. Rev. 4, 35-43.

Waller, P.J. 2006. Sustainable nematode parasite control strategies for ruminant livestock by grazing management and biological control. An. Feed Sci. 126, 277-289.

Waller, P.J., Knox, M.R., Faedo, M. 2001. The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: feeding and block studies with *Duddingtonia flagrans*. Vet. Parasitol. 102, 321-330.

Waller, P.J., Thamsborg, S.M., Larsen, M., Knox, M.R., Peter, R., Miller, J.E., Molento, M.B., Beriajaya, Hood, G.M. 2004. In: Miller, J.E., Waller, P.J. (Eds.), Novel Approaches to Control of Parasites-A Workshop. Vet. Parasitol. 125, 59ï 68.

Wei, J.Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S.C., Aroian, R.V. 2003. *Bacillus thuringiensis* crystal proteins that target nematodes. Natl. Acad. Sci., USA100, 2700-2765.

Zhao, M.L., Huang, J.S., Mo, M.H., Zhang, K.Q. 2005. A potential virulence factor involved in fungal pathogenicity serine-like protease activity of nematophagous fungus *Clonostachys rosea*. Fungal Divers. 19, 217-234.

CHAPTER FOUR

THE EFFECT OF DOSE AND EXPOSURE PERIOD OF SELECTED ISOLATES OF *BACILLUS THURINGIENSIS* (BERLINER) AND *CLONOSTACHYS ROSEA* (SCHROERS) ON THE CONTROL OF GASTROINTESTINAL NEMATODES OF SHEEP

M.A. Baloyi and M.D. Laing

Discipline of Plant Pathology, School of Agricultural Sciences and Agribusiness,

University of KwaZulu-Natal, Private Bag X01, Scottsville 3201, Pietermaritzburg,

South Africa

Abstract

The interaction between inoculum level and time of exposure of selected biocontrol agents (BCAs) on livestock nematodes was investigated in this study. The effect of dosage and time of exposure of *Bacillus thuringiensis* (*Bt*) and *Clonostachys rosea* (Schroers) were studied separately in bioassays conducted in faeces and in water. In a faecal trial different inoculum levels (10^6, 10^8, 10^{10} and 10^{12} spores ml^{-1} for *Bt* and 10^6, 10^8 and 10^{10} conidia ml^{-1} for *C. rosea*) were used while 10^6, 10^8, 10^{10} and 10^{12} spores ml^{-1} for *Bt* and 10^9, 10^{10}, 10^{11}, 10^{12} conidia ml^{-1} for *C. rosea* were used for the water bioassay. In the faecal bioassay, there was no significant interaction between Isolate and Inoculum levels. In the water bioassay, significantly different numbers of surviving nematodes were observed, due to different biological control agents inoculum levels ($P < 0.001$) and Period of Exposure ($P < 0.001$ and $P < 0.012$, for *Bt* and *C. rosea*, respectively), and interactions between inoculum levels and time of exposure ($P < 0.001$ and $P < 0.012$ for *Bt* and *C. rosea*, respectively).

In the faecal bioassay, *Bt* isolate B2 at 10^{10} spores ml^{-1} provided the best control of all the bacterial isolates at all inoculum levels. Isolate P3 was the best fungal isolate at 10^8 conidia ml^{-1} with a control level of 66.6%. In the water bioassay, the *C. rosea* isolate P3 provided 85% control when applied at inoculum levels of 10^9, 10^{10} and 10^{11} conidia ml^{-1}. The importance of time was shown by the increased control of nematodes with time of exposure to the biocontrol agents. Inoculum levels also played a significant part in the activity of the biocontrol agents, with control levels generally increasing with increased spore or conidial doses.

4.1 Introduction

Gastrointestinal nematodes are a serious concern in the small ruminant industry worldwide (Miller and Horohove, 2006). These nematodes cause economic losses due to decreased production, weight loss, reproductive inefficiency, and the cost of treatment and prevention (Barger et al., 1989; McLeod, 1995; Perry and Randolph, 1999; Perry et al., 2002). Anthelmintic

drugs have been the primary method used to combat these effects. However, South Africa and other sheep-producing countries in the world are facing a growing problem of gastrointestinal nematodes in sheep, and the development of anthelmintic resistance is an increasing concern (Prichard, 1994; Waller et al., 1994; Van Wyk et al., 1997; Sangster, 1999; Waller, 2003; Kaplan, 2004).

Use of anthelmintics may result in the presence of drug residues in meat for human consumption, and this adds a further incentive to develop alternative control measures against livestock nematodes (De and Sanyal, 2009).

Most of the research into the biocontrol of livestock nematodes has centered on the nematode-trapping fungus *Duddingtonia flagrans* (Cooke) (Larsen et al., 1992; Knox and Faedo, 2001; Waller et al., 1993; Larsen et al., 1998; Larsen, 2000; Waller et al., 2001; Waller et al., 2004). According to Peña et al. (2002), a dose of 10^5 spores of *D. flagrans* per kg of body weight was effective at reducing infective nematode larvae in faeces from naturally-infected lambs. Results from this experiment showed nematode mortality ranging from 76.6% to 100% after dosing sheep with the fungus for 7d, thus demonstrating the effectiveness of *D. flagrans* on infective larvae of nematodes of lambs. Ghahfarokhi et al. (2004) demonstrated the capability of two nematophagous fungi, *Arthrobotrys oligospora* (Fresen) and *D. flagrans*, to kill *Haemonchus contortus* (Rudolphi) Cobb (Nematoda: Trichostrongylidae) *in vitro* at an inoculum level of 2.0 x 10^4 chlamydospores ml^{-1}.

Bacillus thuringiensis (Berliner) is widely known as a biopesticide in forestry and agriculture and has been identified as a biological control agent for insects (Lacey et al., 2001). This bacterium has a low mammalian toxicity (Hadley et al., 1987; Siegel et al., 2001), and different strains produce target-specific insecticidal products (Schnepf et al., 1998). Kotze et al., (2005) showed that *Bt* strain L366 had nematicidal activity against nematodes.

Clonostachys rosea (Schroers) is a facultative saprophyte which is widely distributed in the soil (Schroers et al., 1999). This fungus was also found to suppress sporulation of the plant pathogen

fungus *Botrytis cinera* (De Bary) and also used for the control of botrytis blight. *C. rosea* showed strong nematicidal activity against *Caenorhabditis elegans* (Maupas).

The primary aim of this study was to find an effective dose and time of exposure of selected bacterial and fungal biocontrol isolates to determine the lowest inoculum level that will give a consistent and high rate of kill the infective stages of gastrointestinal nematodes of sheep.

4.2 Materials and Methods

4.2.1 Spore preparation

B. thuringiensis spores

Spores of three *Bt* isolates, namely, B2(4)M, B10(1)M and B12M were obtained by scraping a frozen culture of bacterial cells that had been stored at -80^{0}C, streaking the cells onto Tryptone Soy Agar, and allowing them to grow for 4d at 25^{0}C. Bacterial colonies were then harvested into an Erlenmeyer flask with 50ml of Tryptone Soy Broth (TSB). These cultures were grown by shaking the flasks in an orbital shaker at 150 rpm at 28^{0}C for 2wks. The cultures were then centrifuged (10 000 rpm at 4^{0}C for 15min) and washed once in 0.1M NaCl and twice in sterile, distilled, ice cold water. The pellets were diluted into sterile, distilled water (40ml) and a standard dilution plate technique was used for calculating the inoculum level of *B. thuringiensis* (as described by Kotze et al., 2005).

Fungal conidia

Conidia of three fungal isolates, namely, Paec1, Paec 3 and Paec 8 were harvested from 3 wk old cultures grown on PDA by scraping the agar surface with a sterile scalpel after addition of 10 ml sterile distilled water. The conidial suspension was filtered through a double layer of cheese cloth into a sterilized 25ml McCartney bottle. The number of conidia in the suspension was determined using a Neubauer haemocytometer under x400 magnification and subsequently adjusted to four inoculum levels (10^6, 10^8, 10^{10} and 10^{12} conidia ml^{-1}) for faecal trial and 10^9, 10^{10}, 10^{11}, and 10^{12} conidia ml^{-1} for water bioassay.

4.2.2 Bioassay in faeces

Faecal samples were collected directly from the rectum of naturally infested sheep that fed on grazing pastures. The sheep were infested with a mixed infection of nematodes, dominated by *Haemonchus contortus* (Rudolphi). The faecal pellets were crushed and mixed thoroughly, and 2 g subsamples were placed in each well of a 24 multiwell microtitre plate. The faeces were sprayed with 2 ml of spore and conidia suspension at different inoculum level (10^6, 10^8, 10^{10} and 10^{12} and 10^6, 10^8 and $10^{10}$for *Bt* and *C. rosea*, respectively) of each of the selected bacterial and fungal isolates (as described by Ghahfarokhi et al., 2004).

4.2.3 Bioassay in water

This technique examined the effect of the biocontrol agents on L3 nematodes extracted into water. The faeces were mixed thoroughly with vermiculite in a 1:1.5 ratio. The mixture was put into a plastic container (100 x 120 x 12.5mm deep), then moistened with sterile distilled water until it was damp but not saturated. The cultures were covered with a lid with holes punched on the top to allow aeration, and incubated at 25^0C for 7d (as described by Graminha et al., 2005). Cultures were kept moist for the entire period by spraying with distilled water when necessary. Following incubation, L3 larval stages were extracted using the Baermann technique (Chandrawathani et al., 1998) and transferred into a 500 ml sterile beaker. The nematodes were surface sterilized with 0.525% sodium hypochlorite solution for 5 min and washed twice with 1L sterile distilled water. One milliliter was pipetted into a counting dish and counted under dissecting microscope. The final inoculum level was adjusted to approximately 35 nematodes ml^{-1}. The nematode suspensions were poured into test tubes and inoculated with spores and conidia of *Bt* and *C. rosea* at levels 10^6, 10^8, 10^{10}, 10^{12}, and 10^9, 10^{10}, 10^{11},10^{12} for *Bt* and *C. rosea*, respectively. Nematodes were counted for 2 d for *Bt* and over 3 d for *C. rosea*.

4.2.4 Statistical analysis

Treatments were arranged in a randomized complete block design, with three replications for each treatment. Both the faecal and the water bioassay were repeated three times.

The following formula was used to determine the nematode mortality (%) by the BCA isolates tested in faeces:

$$\text{Nematode Mortality (\%)} = \frac{[C_1 - C_2]}{C1} \times 100$$

where C_1 = mean number of larvae in untreated manure;

C_2 = number of larvae in treated manure

Data was subjected to factorial ANOVA using GenStat® 11.1 Edition to determine the significance levels of interaction of the main factors (isolate x inoculum level x time). Means of nematode counts were compared using Fisherôs Least Significant Difference (LSD) test at a 5% significance level.

Henderson-Tilton's formula (Henderson and Tilton, 1955) was used to determine the corrected percent mortality in test tubes:

$$\text{Corrected nematode mortality (\%)} = \left(1 - \frac{\text{n in Ctrl before treatment x n in T after treatment}}{\text{n in Ctrl after treatment x n in T before treatment}}\right) \times 100$$

Where n = number of nematodes, Ctrl = control, T = treatment

4.3 Results

4.3.1 Faeces Bioassay

In the faeces bioassay, none of the Interaction Effects were significant between inoculums level and nematode counts (P = 0.43 and P = 0.53 for *B. thuringiensis* and *C. rosea*, respectively). The data is therefore presented as a series of regressions, reflecting the patterns of responses to inoculum levels for the different biocontrol agents, in terms of nematode mortality.

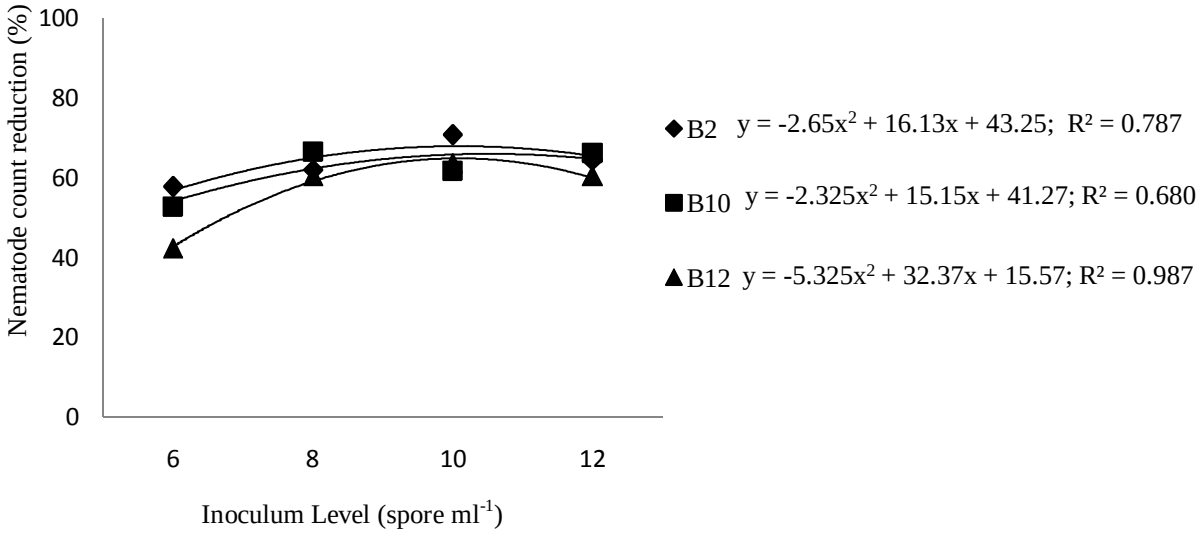

Fig. 4.1: Mortality (%) of nematodes larvae in a faeces bioassay, when treated with three isolates of *B. thuringiensis* at four inoculum levels. Inoculum at a level of 6 = 10^6 spores ml^{-1}, 8 = 10^8 spores ml^{-1}, 10 = 10^{10} spores ml^{-1} and 12 = 10^{12} spores ml^{-1}

The inoculum level of 10^6 spore ml^{-1} was the least effective level at killing nematodes for the three *B. thuringiensis* isolates, with B12 being less effective than B10 and B2. All the *Bt* isolates killed less than 60% of the nematodes when used at the inoculum level of 10^6 spore ml^{-1}. In contrast, at the inoculum levels of 10^8 and 10^{12} spores ml^{-1}, the three *Bt* isolates increased the nematode mortality by 60.3% to 66.1% (Fig. 4.1).

B. thuringiensis Isolate B2 caused the greatest mortality of 70.7% when the inoculum level of 10^{10} spores $ml^{-1}$was used (Fig. 4.1). This isolate was also the best when used at the lowest inoculum level, with a nematode mortality of 57.7%. This isolate consistently maintained a control level of 62%, 70.7% and 64.4% in faeces when applied at inoculum levels of 10^8, 10^{10} and 10^{12} spores ml^{-1}. Isolate B12 was also effective at inoculum levels of 10^8 and 10^{12} spore ml^{-1}, outperforming B2 at these inoculum levels, causing a mortality of 66.5% and 66.1% at 10^8 and 10^{12} spores ml^{-1} respectively.

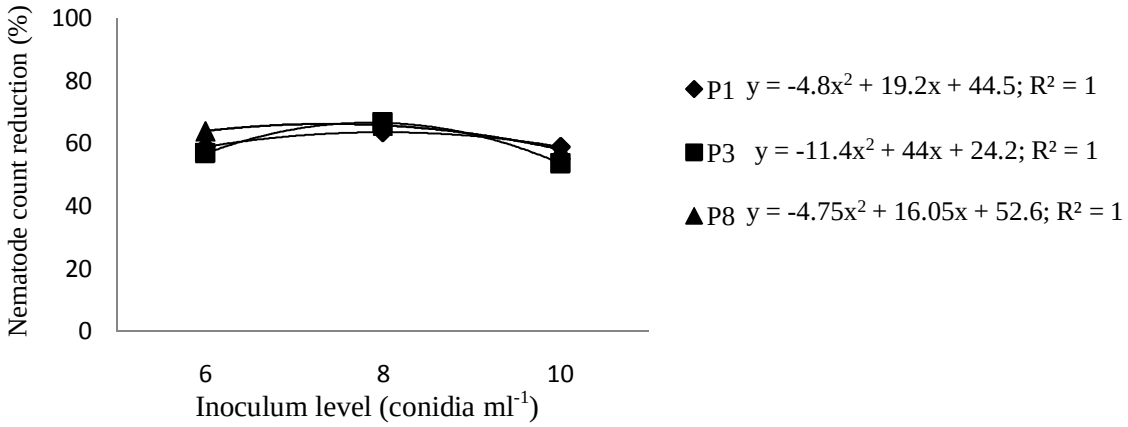

Fig. 4.2: Mortality (%) of nematodes larvae in faeces bioassays treated with three isolates of *C. rosea* conidia at three inoculum levels. Inoculum at a level of 6 = 10^6 conidia ml^{-1}, 8 = 10^8 conidia ml^{-1} and 10 = 10^{10} conidia ml^{-1}

All fungal isolates resulted in nematode mortalities of more than 50% when used at the lowest inoculum level (Fig. 4.2). The single best result was for Isolate P3 at 10^8 conidia ml^{-1}, which caused a mortality of 66.6%. Ironically, this isolate caused the lowest mortality compared to the other isolates when the highest inoculum level of 10^{10} conidia ml^{-1} was applied. All the isolates provided their best control when used at an inoculum level of 10^8 conidia ml^{-1}.

4.3.2 Water bioassay

In the Water Bioassay, there were significant Main Effects (isolates, Inoculum level and time of exposure) and Interaction Effects (isolate x inoculums; isolate x time of exposure; inoculums level x time of exposure and isolate x time of exposure x inoculums level). The data is therefore presented as a table of means, followed by an ANOVA table, identifying the significant F-tests for Main and Interaction Effects.

Table 4.1: Mortality (%) of nematode larvae in a water bioassay, showing a three way interaction between *Bacillus thuringiensis* Isolates x Period of Exposure x *Bt* Inoculum Levels

Period of Exposure (d)	Inoculum levels (per ml)	*Bt* Isolate B2 nematode mortality (%)	*Bt* Isolate B10 nematode mortality (%)	*Bt* Isolate B12 nematode mortality (%)
1	10^6	50.6	26.1	29.5
	10^8	24.5	18.4	8.7
	10^{10}	45.9	68.2	57.6
	10^{12}	35.6	40.5	22.9
2	10^6	27.6	42.5	39.1
	10^8	64.4	70.4	66.0
	10^{10}	53.3	73.3	68.0
	10^{12}	61.7	81.5	90.0

LSD = 14.54; %CV = 20.3

Table 4.2: Factorial ANOVA Table of Main and Interaction Effects of *Bacillus thuringiensis* Isolates x Period of Exposure x Inoculum Levels, from a water bioassay

	DF	F-ratio	P-value	
Main Effects				
Isolate	2	1.96	0.402	NS
Time	1	72.80	< 0.001	***
Inoculum	3	14.82	< 0.001	***
Interaction Effects				
Isolate x Time	2	5.27	0.004	**
Time x Inoculum	3	17.15	< 0.001	***
Isolate x Inoculum	6	1.51	0.145	NS
Isolate x Time x Inoculum	6	1.17	0.241	NS
Residual	46			
Total	71			
CV%	20.3			

* Denotes significantly different at 5% probability
NS denotes not significantly different at 5% probability

Table 4.3: Mortality (%) of nematode larvae in a water bioassay, reflecting a three way interaction between *Clonostachys rosea* Isolates x Period of Exposure x Conidial Inoculum Levels

Period of Exposure (d)	Inoculum	*C. rosea* Isolate P1	*C. rosea* Isolate P3	*C. rosea* Isolate P8
1	10^9	29.6	41.1	32.8
	10^{10}	26.8	36.5	22.3
	10^{11}	32.6	42.8	51.0
	10^{12}	28.3	27.8	46.6
2	10^9	45.4	42.2	55.1
	10^{10}	56.9	48.4	59.9
	10^{11}	59.5	86.9	75.2
	10^{12}	61.1	49.4	57.4
3	10^9	29.3	84.8	72.9
	10^{10}	52.7	84.8	73.0
	10^{11}	55.1	84.8	72.1
	10^{12}	68.5	64.3	82.6

LSD = 13.52; % CV = 17.4

Table 4.4: Factorial ANOVA Table of Main and Interaction Effects of *Clonostachys rosea* Isolates x Period of Exposure x Inoculum Levels, from a water bioassay

	DF	F-ratio	P-value
Main Effects			
Isolate	2	11.91	< 0.001 ***
Time	2	5.54	0.012 **
Inoculum	3	63.01	< 0.001 ***
Interaction Effects			
Isolate x Time	4	4.47	0.003 **
Time x Inoculum	6	2.99	0.012 *
Isolate x Inoculum	6	1.74	0.0125 **
Isolate x Time x Inoculum	12	1.65	0.098 NS
Residual	70		
Total	107		
CV%	17.4		

* Denotes significantly different at P Ò0.05
NS denotes not significantly different at P Ò0.05

Isolate

The three *Bt* isolates were not significantly different in their ability to kill nematodes in the water bioassay when applied alone (P = 0.402). In contrast, there was a significant difference between isolates of *C. rosea* in their ability to kill nematodes (P < 0.001).

Period of exposure

The Period of Exposure had a significant effect on nematode mortality (P < 0.001 for *Bt* and P = 0.012 for *C.rosea*) for all the BCAs. Mortality on Day 1 was always less than on other days, for all BCAs, as was expected. For *Bt,* on Day 1 control levels reached 35.6%. This increased to 61.7% on Day 2. Similarly, *C. rosea* isolates reduced nematode counts by 45.4%, 42.2%, and 55.1% for Isolates P1, P2 and P8 respectively by Day 2. The control level increased further to 84.8% and 72.9% for isolates P2 and P3, respectively. However, isolate P1 showed a lower mortality of 29.3% by Day 3.

Inoculum level

Inoculum Levels also had a significant effect on the mortality of nematodes in the water bioassay. Nematode mortality was least when the lowest inoculum level was used for both *Bt* and *C. rosea.* In general, nematode mortality increased with increasing inoculum level. However, this changed when the inoculum level was increased to the highest inoculum levels of 10^{12} spores or conidia ml^{-1}, for isolates of *Bt* and *C. rosea*, respectively (Fig. 4.3 and Fig. 4.4). The best inoculum levels were 10^{10} spores ml^{-1} for the *Bt* isolates (Fig 4.3) and 10^{11} conidia ml^{-1} for *C. rosea* isolates (4.4).

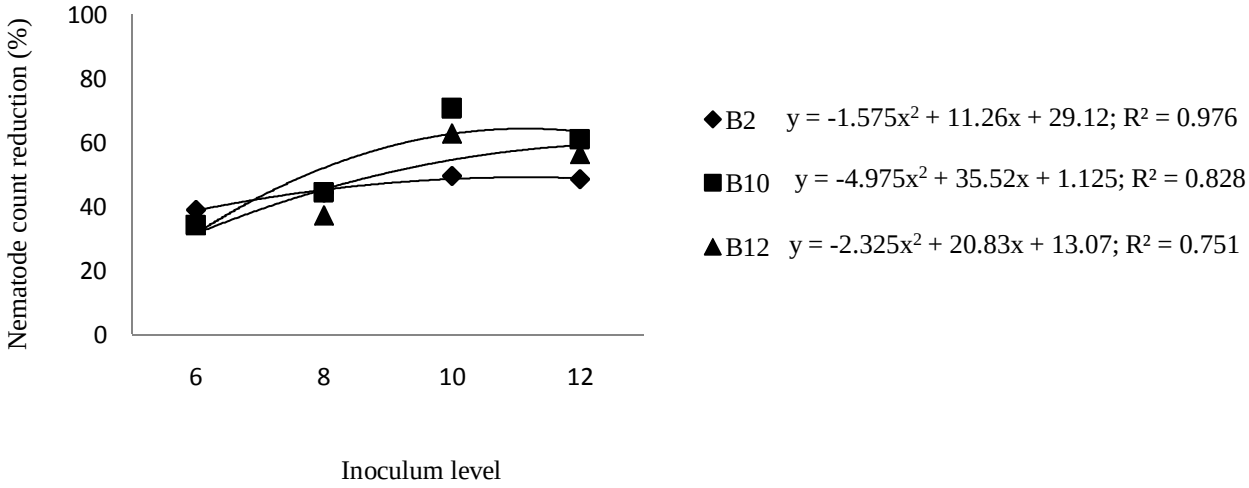

Fig. 4.3: Mortality (%) of nematode larvae in a water bioassay when treated with three *B. thuringiensis* isolates at four inoculum levels. Inoculum at a level of 6 = 10^6 spores ml^{-1}, 8 = 10^8 spores ml^{-1} 10 = 10^{10} spores ml^{-1} and 12 = 10^{12} spores ml^{-1}

There was no significant interaction between *Bt* isolates and Inoculum levels (P = 0.145). Isolate B2 provided relatively poor control at all inoculum levels. Isolate B10 killed 70.8% of the nematodes when an inoculum level of 10^{10} spores ml^{-1} was used.

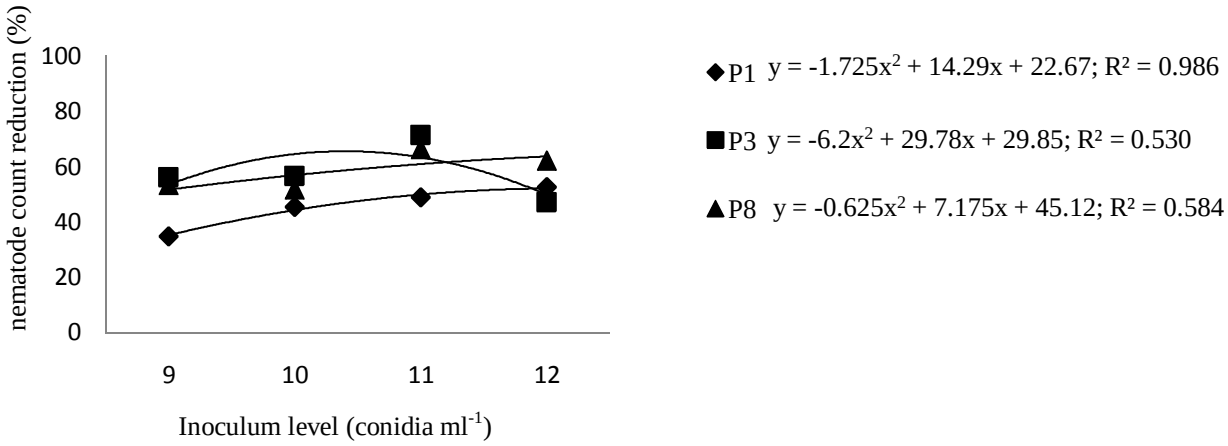

Fig. 4.4: Mortality (%) of nematode larvae in a water bioassay when treated with three *C. rosea* isolates at four inoculum levels. Inoculum at a level of 9 = 10^9 conidia ml^{-1}, 10 = 10^{10} conidia ml^{-1} 11 = 10^{11} conidia ml^{-1} and 12 = 10^{12} conidia ml^{-1}

Interaction of isolate x period of exposure

There was a significant interaction between Isolate and period of exposure, resulting in increasing nematode mortality when *Bt* was used (P = 0.004). All isolates caused the least control on Day 1 and nematode mortality increased on Day 2. Isolate B2 was the most effective on Day 1, but was the least effective on Day 2. Isolate B12 was the most effective isolate on Day 2.

There was a significant interaction between *C. rosea* isolates and period of exposure (P = 0.003) (Table 4.4). The nematode mortality was low on Day 1 for isolates Paec 1 and Paec 3, but this increased slightly in Day 2. Isolates Paec 3 andPaec 8 provided higher control levels in Day 3 than Paec 1. Paec 3 was the best isolate on Day 3 with a mortalityof 84.8%.

Interaction of period of exposure x inoculum level

There was a significant interaction between Period of Exposure and Inoculum Level ($P < 0.001$) for the *Bt* isolates. Control of nematodes was similar in both Day 1 and Day 2 when the lowest inoculum level was used. The difference in nematode mortality was only 1% between the two days. As inoculum levels increased to 10^8 spores ml^{-1}, the difference in control levels between the two days increased by 49.8%. At the inoculum level of 10^8 spores ml^{-1}, nematode mortality increased to a gap of 17.2% to 67.0% for Day 1 and Day 2, respectively. When 10^{10} spores ml^{-1} was used, control also differed between the days from 57.0% on Day 1 to 64.9% on Day 2. Moreover, this inoculum level resulted in the best level of control on Day 1. At the highest inoculum level of 10^{12} spores ml^{-1} a lower level of mortality occurred on Day 1, and then increased to 77% on Day 2.

For *C. rosea* there was also a significant interaction between period of exposure and inoculum level ($P = 0.012$). Inoculum levels of 10^9, 10^{10} and 10^{12} conidia ml^{-1} killed more nematodes as the period of exposure increased. Inoculum levels of 10^{10} and 10^{12} conidia ml^{-1} cause a similar nematode mortality on Day 3. At an inoculum level of 10^{11} conidia ml^{-1} the highest level of mortality occurred on Day 2. On Day 3, nematode mortality was similar for inoculum levels of 10^{10}, 10^{11} and 10^{12} conidia ml^{-1}.

Three way interaction of BCAs

There were no significant three ways interactions for either the *Bt* or the *C. rosea* isolates. However, the single most effective treatment with *Bt* was with Isolate B12 at the highest inoculum level (10^{12} spores ml^{-1}) on Day 2 and Isolate P8 at 1012 conidia ml-1 on Day 3.

.

4.4 Discussion

Mortality of nematodes caused by *B. thuringiensis* isolates varied as a function of the inoculum level used, with a very strong relationship between *Bt* inoculum levels and mortality reflected in both bioassays. Although the highest inoculum level of 10^{12} spores ml^{-1} was the most effective for Isolates B10 and B12, it may not be the most cost-effective because the cost of production may be higher and will increase the costs of application for livestock farmers. Interestingly, Isolates B10 and B12 used at 10^{8} spore ml^{-1} reduced the number of nematodes by 70.4% and 66.0% on Day 2, in the water bioassay, whereas at 10^{12} spore ml^{-1} the mortalities were 81.5% and 90.0%, respectively. In other words, for an increase of 11.1% and 24.0% in nematode mortality, 10,000 times as many spores of *Bt* had to be applied.

C. rosea did not seem to respond as much to inoculum level in order to achieve much higher mortality rates. At an inoculum level of 10^{8} conidia ml^{-1}, P3 was able to kill more nematodes than it did at a higher inoculum level of 10^{12} conidia ml^{-1}. All isolates resulted in a nematode mortality of more than 50% at the lowest inoculum level.

Control of nematodes was highly significant at different inoculum levels ($P < 0.001$) and on different days ($P < 0.001$) for both *Bt* and *C. rosea*. Further more, *C. rosea* isolates also showed significantly different effect in controlling nematodes ($P < 0.001$). This means that the BCAs activity against nematodes was influenced by exposure period, inoculum level and isolates in the case of *C. rosea*.

Exposure period has an important role in the control of nematodes. Generally, low activity against nematodes was observed on the first day. However, this increased on the second day. This suggests that the activity of the biocontrol agent is not immediate. Peña et al. (2002) also reported similar results of larval development being significant between doses from Day 2. However, Mendoza de Gives et al. (1998) showed that *D. flagrans* killed nematodes within 14 h. The low activity of BCAs on Day 1 reflects the need for nematodes to consume *Bt* spores before being killed, and that may take some time. The importance of time was also observed by OôGrady et al. (2007) who suggest that it would be best to use *B. thuringiensis* L366 to control livestock nematodes by administering it early to allow it to germinate and grow through a vegetative phase,

followed by sporulation and production of *Cry* proteins, before any anthelmintic effect could be observed.

Inoculum level is also of great importance in controlling nematodes. This is shown by the positive response of isolates to increasing inoculum levels. Isolates of *Bt* achieved satisfactory results when 10^{10} spores ml^{-1} were used and further increase in control resulted from increases in inoculum levels for isolates B10 and B12.

Use of lower inoculum levels of 10^9 and 10^{10} conidia ml^{-1} for *C. rosea* resulted in satisfactory control of nematodes. However, an inoculum of 10^{11} conidia ml^{-1} provided better level of nematode control. A higher inoculum level of 10^{12} conidia ml^{-1} did not result in increased control.

Isolate B10 killed nematodes faster than the other isolates. Although Isolate B2 killed higher number of nematodes on Day 1 than the other isolates, its activity did not increase much on the second day. Better control was provided by the fungal isolates on Day 3, with P3 and P8 responding strongly to an increase in period of exposure. Isolate P1 did not increase its activity with increasing periods of exposure.

When *Bt* spores were used, the inoculum level influenced the speed of kill. The highest inoculum level resulted in the highest level of control of nematodes within 2 days. Inoculum level of fungal conidia did not influence the speed of kill. This study agrees with Maddox et al. (2000) who found that many species of fungi that produced microsporidia did not immediately kill their insect or nematode hosts, but often caused death in later developmental stages.

In conclusion, for both biocontrol agents, *Bt* and *C. rosea*, the inoculum level and period of exposure are important parameters that affect the performance of the biocontrol agents. An inoculum level of 10^8 spore ml^{-1} for the three *Bt* isolates was enough to effectively reduce the number of nematodes in faeces. In contrast, high levels of inoculum were not as important for the *C. rosea* isolates, especially Isolate P3, which should make its application economically attractive. For example, at a level of 10^9 conidia ml^{-1} Isolate P3 killed 85% of the nematodes within three days.

4.5 References

Barger, I.A. 1989. Helminth parasites and animal production. In, Biology and Control of Endoparasites. (Ed.) Symonds, L.E.A. Donald, A.D., Dineen, J.K. Academic Press, New York.

Chandrawathani, P., Omar, J., Waller, P.J. 1998 The control of the free-living stages of *Strongyloides papillosus* by the nematophagous fungus, *Arthrobotrys oligospora*. Vet. Parasitol. 76, 321ï 325.

De, S., Sanyal, P.K. 2009. Biological control of helminth parasites by predatory fungi. Vetscan 4, 1-9.

Ghafarokhi, M.S., Abyaneh, M.R., bahadori, S.R., Eslami, A., Zara, R., Ebrahimi, M. 2004. Screening of soil and sheep faecal samples for predacious fungi: Isolation and characterization of the nematode-trapping fungus *Arthrobotrys oligospora*. Iran. Biomed. J. 8, 135-142.

Graminha, E.B.N., Costa, A.J., Oliveira, G.P., Monteiro, A.C., Palmeira, S.B.S. 2005. Biological control of sheep parasite nematodes by nematode-trapping fungi: *in vitro* activity and after passage through gastrointestinal tract. World J. Micro. Biotech. 21, 717-722.

Hadley, W.M., Scott, W.B., McDowell, T.D., Thilsted, J.P., Hibbs, C.M., Whorton, J.A., Day, P.W., Friedmann, M.B., Stoll, R.E. 1987. Five months oral (Diet) toxicity/infectivity study of *Bacillus thuringiensis* insecticides in sheep. Fund. Appl. Toxicol. 8, 236-242.

Henderson, C.F., Tilton, E.W. 1955. Tests with acaricides against the brown wheat mite. J. Econ. Ento. 48, 157-161.

Kaplan, R.M. 2004. Drug resistance in nematodes of veterinary importance: A status report. Trends Parasitol. 20, 477-581.

Knox, M.R., Faedo, M. 2001. Biological control of field infections of nematode parasites of young sheep with *Duddingtonia flagrans* and effects of spore intake on efficacy. Vet. Parasitol. 101, 155-160.

Kotze, A.C., OõGrady, J., Gough, J.M., Pearson, R., Bagnall, N.H., Kemp, D.H. Akhurst, R.J. 2005. Toxicity of *Bacillus thuringiensis* to parasitic and free-living life-stages of economically important nematode parasites of livestock. Int. J. Parasitol. 35, 1013ï 1022.

Lacey, L.A., Frutos, R., Kaya, H.K., Vale, P. 2001. Insect pathogens as biological control agents: do they have future? Biol. Control 21, 230-248.

Larsen, M. 2000. Prospects for controlling animal parasitic nematodes by micro fungi. Parasitology 120, S120-S131.

Larsen, M., Faedo, M., Waller, P.J., Hennessy, D.R. 1998. The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: Studies *Duddingtonia flagrans*. Vet. Parasitol. 76, 121-128.

Larsen, M., Nansen, P., Gronvold, J., Wolstrup, J., Henriksen, S.A. 1992. *In vivo* passage of nematophagous fungi selected for biocontrol of parasitic nematodes in ruminants. J. Helminth. 66, 137-141.

Maddox, J.V., Brooks, W.M., Solter, L.F. 2000. Bioassay of microsporidia. In, Bioassays of Entomopathogenic Microbes and Nematodes, (Ed.) Navon, A., Ascher, K.R.S. CABI Publishing, New York.

McLeod, R.S. 1995. Costs of major parasites of the Australian livestock industries. Int. J. Parasitol. 25, 1363-1367.

Mendoza de Gives, P., Flores Crespo, J., Herrera Rodriguez, D., Vazquez Prats, V., Liebano Hernandez, G.E. 1998. Biological control of *Haemonchus contortus* larvae in ovine faeces by administering an oral suspension of *Duddingtonia flagrans* chlamydospores to sheep. J. Helminth. 72, 343-347.

Miller, J.E., Horohove, D.W. 2006. Immunological aspects of nematode parasite in sheep. J. An. Sci. 84, E124-E132.

OõGrady, J., Akhurst, R.J., Kotze, A.C. 2007. The requirement for early exposure of *Haemonchus contortus* larvae to *Bacillus thuringiensis* for effective inhibition of larval development. Vet. Parasitol. 150, 97-103.

Peña, M.T., Miller, J.E., Fontenot, M.E., Gillipie, A., Larsen, M. 2002. Evaluation of *Duddingtonia flagrans* in reducing infective larvae of *Haemonchus contortus* in faeces of sheep. Vet. Parasitol. 103, 259-265.

Perry, B.D., Randolph, T.F. 1999. Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. Vet. Parasitol. 84, 145-168.

Perry, B.D., Randolph, T.F., McDermott, J.J., Sones, K.R., Thornton, P.K. 2002. Investing in Animal Health Research to Alleviate Poverty. ILRI (International Livestock Research Institute), Nairobi, Kenya, 148pg.

Prichard, R.K. 1994. Anthelmintic resistance. Int. J. Parasitol. 54, 259-268.

Sangster, N.C. 1999. Anthelmintic resistance: Past, present and future. Int. J. Parasitol. 29, 115-124.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zigler, D.R., Dean, D.H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Micro. Mol. Biol. Rev. 62, 775-806.

Schroers, H.J., Samuels, G.J., Seifert, K.A., Gams, W. 1999. Classification of the mycoparasite *Clonostachys rosea* in *Clonostachys* as *C. rosea*, its relationship to *Bionectria ochroleuca*, and notes on other *Gliocladium*-like fungi. Mycologia 91, 365ï 385.

Siegel, J.P. 2001. The mammalian safety of *Bacillus thuringiensis*-based insecticides. J. Invert. Path. 77, 13-21.

Van Wyk, J.A., Malan, F.S., Randles, J.L. 1997. How long before resistance makes it impossible to control some field strains of *Haemonchus contortus* in South Africa with any of the anthelmintics? Vet. Parasitol. 70, 111-122.

Waller, P.J. 1994. The development of anthelmintic resistance in ruminants livestock. Acta Trop. 56, 233-243.

Waller, P.J. 2003. Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy, with emphasis on biological control. An. Health Res. Rev. 4, 35-43.

Waller, P.J., Faedo, M. 1993. The potential of nematophagous fungi to control the free-living stages of nematode parasites: *In vitro* and *in vivo* studies. Vet. Parasitol. 51, 289-299.

Waller, P.J., Knox, M.R., Faedo, M. 2001. The potential of nematophagous fungi to control the free-living stages of sheep: Feeding and block studies with *Duddingtonia flagrans*. Vet. Parasitol. 102, 321-330.

Waller, P.J., Schwan, O., Ljungstrom, B.L., Rydzik, A., Yeates, G.W. 2004. Evaluation of biological control of sheep parasites using *Duddingtonia flagrans* under commercial farming conditions on the island of Gotland, Sweden. Vet. Parasitol. 126, 299-315.

CHAPTER FIVE

USE OF MIXED CULTURES OF BIOCONTROL AGENTS TO CONTROL SHEEP NEMATODES

M.A. Baloyi and M.D. Laing

Discipline of Plant Pathology, School of Agricultural Sciences and Agribusiness,

University of KwaZulu-Natal, Private Bag X01, Scottsville 3201, Pietermaritzburg,

South Africa

Abstract

Biological control is a promising non-chemical approach for the control of gastrointestinal nematodes of sheep. Use of combinations of biocontrol agents have been reported to be an effective method to increase the efficacy of biological control. In this study, combinations of either two *Bt* or *C. rosea* isolates and *Bt*+*C. rosea* isolates were evaluated *in vitro* in microtitre plates for their biocontrol activity on sheep nematodes. Results indicate that there was a significant reduction of nematode counts due to a combination of biocontrol agents ($P < 0.001$). Combinations of *Bt* isolates reduced nematodes counts by 72.8%, 64% and 29.8% for combinations B2+B12, B2+B10 and B10+B12. The results revealed a control level of 57% when *C. rosea* isolates P3+P8 were combined. Combinations of *Bt* and *C. rosea* isolates B10+P8 caused the greatest mortality of 76.7%. Most combinations were antagonistic (B10+B12, B12+P8, B2+P8, B10+P3, B2+P3, B12+P3 and P3+P8), with only a few combinations showing an additive effect (B2+B10, B2+B12 and B10+P8). None were synergistic.

5.1 Introduction

Parasitic nematode infections are generally regarded as the most prevalent health problem of small ruminants worldwide, causing significant production losses (Perry and Radondolph, 1999). Although anthelminthic drugs have been a reliable control method, resistance by nematodes to these drugs has become a serious threat to sheep production (Prichard, 1994; Waller, 1994; Van Wyk et al., 1997; Sangster, 1999; Waller, 2003; Kaplan, 2004).

A number of microorganisms have been found to feed on nematodes, reducing the number of free-living larvae in the soil ecosystem (Stirling, 1991; Chen and Dichinson, 2004) in a process known as biological control. Amongst these microorganisms, the genera *Duddingtonia, Arthrobotrys, Nematoctonus* and *Monacrosporium* have shown their capability to kill and digest animal parasitic nematodes, which reflects their potential as biocontrol agents (Dimander et al., 2003; Campos et al., 2008).

Kotze et al. (2005) showed that several isolates of the bacterium *Bacillus thuringiensis* (*Bt*) (Berliner) are nematicidal to larvae of *Haemonchus contortus* (Rudolphi), *Trichostrongylus colubriformis* (Giles) and *Ostertagia circumcincta* (Stadelmann), which are nematodes of livestock. Isolates of this bacterium produce highly specific insecticidal crystal proteins, which are widely used for the control of pests in agriculture.

McPartland (2000) discovered that *Clonostachys rosea* (Schroers) was compatible when combined with *Trichoderma harzianum* (Rifai). Many isolates of *C. rosea* are highly efficient antagonists against several plant pathogenic fungi and soil fungi (Gan et al., 2007). This fungus is known to produce serine protease and chitinase as its key mode of action against fungi and nematodes of plants (Zhao et al., 2005; Li et al., 2006).

Both *Bt* and *C. rosea* have shown their ability to reduce nematode populations. However, most biocontrol agents have been inconsistent in the field. Inconsistency of performance of the biocontrol agents is influenced by both biotic and abiotic factors. Biotic factors include interactions with non-target organisms, varying rhizosphere or soil colonization by the biocontrol agent, varying initial population levels and the genetic diversity of target pathogens (Stirling, 1991; Pierson and Waller, 1994; Meyer and Roberts, 2002).

In this regard, combinations of biocontrol agents may have more potential to suppress pathogens due to their different traits responsible for parasitism (Pierson and Waller, 1994; Crump, 1998). Literature on the effect of combining biocontrol agents on crops is well documented. However, there are no references on combining biocontrol agents for the control of livestock nematodes.

The aim of this study was to evaluate the effect of combining *Bt* and *C. rosea* for the control of livestock nematode populations when mixed in a culture of sheep faeces.

5.2 Materials and Methods

Biological control agents

The isolates of *Bt* and *C. rosea* were isolated from pasture soils of the livestock section at Ukulinga Research Farm of the University of KwaZulu-Natal, Pietermaritzburg, as described in Chapter 2.

5.2.1 Preparation of biocontrol agents

Spores of *B. thuringiensis*

Spores of *Bt* were obtained by scraping a frozen culture that was stored at -80^{0}C and streaking cells onto Tryptone Soy Agar plates and kept for 4d at room temperature. The cells were then cultured in an Erlenmeyer flask containing 50ml of Tryptone Soy Broth, incubated and shaken at 150 rpm at 28^{0}C for 2wks. After incubation, the cultures were centrifuged (10 000 rpm at 4^{0}C for 15min), washed once in 0.1 mole (M) NaCl and twice in sterile distilled ice cold water. The pellets were diluted into 9ml of sterile distilled water. The number of bacterial spores was determined using a dilution plate technique and adjusted to 10^8 spores $m1^{-1}$.

Conidia of *C. rosea*

Conidia were produced by growing the fungal cultures on PDA for 3wks. Spores were recovered from the fungal culture by washing the surface of the agar with sterile distilled water. The conidial suspension was filtered through a double layer of cheese cloth into a sterilized beaker. The suspension of conidia was counted with a haemocytometer and adjusted to 10^6 conidia ml^{-1}.

5.2.2 Faecal Bioassay of mixed culture of biocontrol agents

To conduct the bioassay, faecal samples were collected directly from the rectum of naturally infested sheep that feed on grazing pastures. The sheep had a mixed infection of nematodes. The faecal pellets were broken up and mixed thoroughly, and then a subsample of 2g of the faeces were placed in each well of a 24 multiwell microtitre plate and treated with a spore or conidial suspension at a ratio of 1:1 (w/v) as described by Ghahfarokhi et al. (2004) with minor modifications. The isolates were used individually and in combinations that consisted of the same genus (i.e. mixture of two *Bt* or *C. rosea* isolates) and different genera (i.e. mixture of *Bt* and *C.*

rosea). The treated samples were incubated at 25^0C for 7d. The surviving nematode larvae were recovered using the Baermann technique.

5.2.3 Statistical Analysis

Experiments were repeated twice, with treatments arranged in a randomized complete block design (RCBD). Data were subjected to analysis of variance (ANOVA) using the Genstat® 11^{th} edition statistical package. Fisherôs Least Significant Difference (LSD) was used to determine differences between treatment means. The following formula was used to determine the mortality of nematodes (Abbott, 1925).

$$\text{Nematode mortality (\%)} = \frac{[C_1 - C_2]}{C_1} \times 100$$

Where C_1 = mean number of larvae in untreated manure;

C_2 = number of larvae in treated manure

In order to determine the combined effects of *Bt* and *C. rosea* isolates, the observed mortality levels were compared to the expected mortality rate under the assumption of an independent effect, as described by Jazzar and Hammad (2004).

For example, the expected mortality of the combination B2+P8 for the *Bt*: Isolate B2 and *C. rosea* Isolate P8 was calculated using the following formula:

$$B2+P8_{(Expected)} = B2 + P8\ (1-B2)$$

Where B2 and P8 are the observed proportion mortality caused by *Bt* and *C. rosea* isolates alone.

The same calculation was used for all the other combinations.

After calculating the G^2 the results were compared to the table (df = 1, P Ò0.05)

$$G^2 = (O - E)^2 / E$$

Where *O* is the observed mortality for either of the isolates and *E* is the expected mortality for either of the isolates. Additivity would be indicated by the $\chi^2 < 3.84$, whereas synergistic or antagonistic interactions would be indicated by the $G^2 > 3.84$. If $O < E$, the interaction would be considered antagonist, whereas if $O >$ E then the interaction was synergistic (Finny, 1964).

5.3 Results

5.3.1 Effect of individual and mixed culture application of biocontrol agents in faeces

Individual application of biocontrol agents caused expected levels of mortality of nematodes. The best isolate was the fungal Isolate P3, which caused a control level of 66.3% of nematodes, followed by P8, which caused a reduction of 54.0% (Table 5.1). The *Bt* isolates caused control levels of 52.0%, 48.7% and 47.0%, for Isolates B12, B10 and B2, respectively.

Mixing of biocontrol agents caused a significant interaction effect ($P < 0.001$). The combination of isolates from the same genus affected nematode mortality in various ways. Combinations of bacterial isolates caused mortality of 64% and 29.8% for Isolates B2+B10 and Isolates B10+B12, respectively. The combination of fungal Isolates P3 and P8 caused a control level of 57%. The results revealed a difference in mortality of 15% when comparing the effects of mixing two fungal isolates and two bacterial isolates.

The best combination of *Bt* and *C. rosea* isolates was B10+P8 which caused a nematode mortality of 76.7%. This combination caused the greatest mortality of nematodes when compared to all the other combinations, and had a great effect than when they were applied individually.

Table 5.1: Performance of individual and mixed biocontrol agents in controlling livestock nematodes in sheep faeces

Isolate	Mean mortality (%)	Transformed mean mortality	Rank
B10+B12	29.8	30.6**a**	14
B12+P8	44.3	41.7**ab**	13
B2+P8	46.2	42.7**ab**	12
B10+P3	46.5	42.7**ab**	11
B2	47.0	43.3**ab**	10
B10	48.7	44.2**ab**	9
B12	52.0	46.2**b**	8
B2+P3	53.5	47.0**bc**	7
Paec8	54.0	47.3**bc**	6
B12+P3	57.0	49.0**bc**	5
P3+P8	57.0	49.0**bc**	5
B2+B10	64.0	53.2**bc**	4
Paec3	66.3	54.6**bc**	3
B2+B12	72.8	59.4**c**	2
B10+P8	76.7	61.8**c**	1
F-ratio		10.81	
P-value		<0.001***	
LSD		12.31	
CV%		16.6	

Means followed by the same letter are not significantly different (P > 0.05)

Values are means of three replicate experiment repeated two times

* Denotes significantly different at 5% probability

When Isolate P8 isolate was applied with the *Bt* isolates, the combinations resulted in a control lower than 50%. Although Isolate P3 alone caused a nematode reduction of 66.3% control was not improved when combined with other *Bt* and *C. rosea* isolates.

Table 5.2: Effect of combining *Bacillus thuringiensis* and *Clonostachys rosea* in controlling livestock nematodes in faeces

Treatment	**Observed%**	**Expected%**	χ^2	**Interaction effect**
B10+B12	29.8	75.376	27.55747	Antagonistic
B12+P8	44.3	77.92	14.50596	Antagonistic
B2+P8	46.2	75.62	11.44587	Antagonistic
B10+P3	46.5	82.7119	15.85385	Antagonistic
B2+P3	53.5	82.139	9.985419	Antagonistic
B12+P3	57.0	83.824	8.583782	Antagonistic
P3+P8	57.0	84.498	8.948614	Antagonistic
B2+B10	64.0	72.811	1.066236	Additive
B2+B12	72.8	74.56	0.041545	Additive
B10+P8	76.7	76.402	0.001162	Additive

The combination of biocontrol agents resulted in two different interactions, most of which were antagonistic, although some combinations were additive (Table 5.2). None of the combinations were synergistic.

5.4 Discussion

All the isolates controlled nematodes effectively when applied alone. Individual application of *C. rosea* isolates caused greater mortality of nematodes than the bacterial isolates. Isolate P3 was the best isolate and ranked third, compared to both individual and combination applications of other *C. rosea* isolates (Table 5.1).

The performance of Isolate P3 did not improve when combined with other bacterial and fungal isolates. The interaction effect of combining P3 with all the bacterial isolates and the fungal Isolate P8 was antagonistic. Combination of bacterial Isolates B10+B12 also had a negative interaction. Other researchers have reported similar cases of negative interaction between biocontrol agents (Meyer and Roberts, 2002). This might be due to the competition between biological control agents (McVay et al., 1977), or biochemical substances produced that may interfere with the action of the other biocontrol agents (Fravel, 1988). This indicates that, although they are species in the same genus, they may exhibit a certain degree of defensiveness against members of their own genus or species (Alabouvette and Lemanceau, 1998). Meyer and Roberts (2002) identified the possibility of biocontrol agents mechanisms being also directed to the companion biocontrol agent within the combination. Guetsky et al. (2002) noted the possibility of one mechanism to compensate for the other mechanism when multiple mechanisms are involved under a certain set of conditions.

The combination of the bacterial Isolate B10 and the fungal Isolate P8 was additive in its control of nematodes. This may be due to the different modes of action involved because *C. rosea* is known to target nematodes by producing protease and chitinase enzymes (Zhao et al., 2005). On the other hand, *Bt* is known to produce endotoxins that directly target free-living and adult larval stages after ingestion (Kotze et al., 2005). Moreover, combining isolates B2 with B10 improved their efficacy in controlling nematodes more than each of the isolates used alone.

In this study, no combinations resulted in a synergistic interaction. To produce a synergistic effect, the combined effect of biological control agents must be greater than their additive individual effects (Ansari et al., 2008), which was not the case in this study. Some authors have reported the synergistic effect of combining biological agents, reflecting the interactive advantage of combining the biocontrol agents (Alabouvette et al., 1996). Hence, selection of biocontrol agents that have different modes of action can enhance biocontrol activity.

A combination of biological control agents may be considered in cases where the isolate combination has the ability to kill more nematodes than when applied alone. In this study, only three combinations may be recommended, two of them bacterial and one of bacterial and fungi (B2+B10; B2+B12 and B10+P8).

In conclusion, combining bacterial isolates of the same genus from the same ecology may have an advantage in controlling nematode counts. Also, biological control agents from different genera with different modes of action that target different stages in the lifecycle of the target organism may increase the control of nematodes. However, in this study most isolates were antagonistic towards other isolates, irrespective of the genera. Thus, a generalized claim that combining biological control agents will be beneficial is not always true.

5.5 References

Abbott, W.S. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Ento. 18, 265ï 267.

Alabouvette, C., Lemanceau, P. 1998. Joint action of microbials for disease control. In: Biopesticides: Use and Delivery. Hall, F.R., Menn, J.J. (Eds.), Humana Press, Ootowa, Canada.

Alabouvette, C., Lemanceau, P., Steinberg, C. 1996. Biological control of *Fusarium* wilts: opportunities for developing a commercial product. In: Hall, R. (Ed.) Principles and Practice of Managing Soilborne Plant Pathogens, APS Press, St Paul, United States. pp. 192ï 212.

Ansari, M.A., Shah, F.A., Butt, T.M. 2008. Combined use of entomopathogenic nematodes and *Metarhizuim anisopliae* as a new approach for black vine weevil, *Otiorhynchus sulcatus*, control. Ent. Exp. Appl. Vol. 129, 340-347.

Campos, A.K., Arau´jo, J.V., Guimaraěes, M.P. 2008. Interaction between the nematophagous fungus *Duddingtonia flagrans* and infective larvae of *Haemonchus contortus* (Nematoda: Trichostrongyloidea). J. Helminthol. 82, 337ï 341.

Chen, S., Dickinson, D.W. 2004. Biological control of nematodes by fungal antagonists. In: Chen, Z., Chen, S., Dickinson, D.W. (Eds.), Nematology-Advances and Perspectives, Vol. II. Nematode Management and Utilization. Tsinghua University Press and CABI Publishing, Beijing, China and Wallingford, UK.

Crump, D.H. 1998. Biological control of potato and beet cyst nematodes. Ann. Appl. Biol. 53, 383ï 386.

Dimander, S.O., Hoglund, J., Uggla, A., Sporndly, E., Waller, P.J. 2003. Evaluation of gastro-intestinal nematode parasite control strategies for first-season grazing cattle in Sweden. Vet. Parasitol. 111, 192ï 209.

Finney, D.J. 1964. Probit Analysis. Cambridge University Press, London, UK.

Fravel, D.R. 1988. Role of antibiosis in the biocontrol of plant diseases. Ann. Rev. Phytopath. 26, 75-91.

Gan, Z., Yang, J., Tao, N., Yu, Z., Zhang, K. 2007. Cloning and expression analysis of a chitinase gene *Crchi1* from the mycoparasitic fungus *Clonostachys rosea* (syn. *Gliocladium roseum*). J. of Micro. 45, 422-430.

Ghahfarokhi, M.S., Abyaneh, M.R., Bahadori, S.R., Eslami, A., Zare, R., Ebrahimi, M. 2004. Screening of soil and sheep faecal samples for predacious fungi: Isolation and characterization of the nematode-trapping fungus *Arthrobotrys oligospora.* Iran. Biomed. J. 8, 135-142.

Guetsky, R., Shtienberg, D., Elad, Y., Fischer, E., Dinoor, A. 2002. Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. Phytopathology 91, 621-627.

Jazzar, C., Hammad, A.F. 2004. Efficacy of multiple biocontrol agents against the sweet potato whitefly *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) on tomato. J. Appl. Ento. 128, 188-194.

Kaplan, R.M. 2004. Drug resistance in nematodes of veterinary importance: A status report. Trends Parasitol. 20, 477-581.

Kotze, A.C., O'Oðrady, J., Gough, J.M., Pearson, R., Bagnall, D.H., Akhurst, R.J. 2005. Toxicity of *Bacillus thuringiensis* to parasitic and free-living life stages of nematode parasitic of livestock. Int. Parasitol. 35, 1013-1022.

Li, J., Yang, J.K., Huang, X.W., Zhang, K.Q. 2006. Purification and characterization of an extracellular serine protease from *Clonostachys rosea* and its potential as a pathogenic factor. Process Biochem. 41, 925-929.

McPartland, J. 2000. Biological control of hemp. Third In. Symposium: Bioresource hemp. (Wolfsburg, Germany).

McVay, J.R., Gudauskas, R.T., Harper, J.D. 1977. Effects of *Bacillus thuringiensis* nuclear-polyhydrosis virus mixtures on *Trichoplusiani* larvae. J. Invert. Path. 29, 367-372.

Meyer, S.L.F., Roberts, D.P. 2002. Combinations of biocontrol agents for management of plant-parasitic nematodes and soilborne plant-pathogenic fungi. J. Nematol. 34, 1ï 8.

Perry, B.D., Randolph, T.F. 1999. Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. Vet. Parasitol. 84, 145-168.

Pierson, E.A., Waller, D.M. 1994. Use of mixtures of fluorescent pseudomonads to suppress take-all and improve the growth of wheat. Phytopathology 84, 940ï 947.

Prichard, R.K. 1994. Anthelminthic resistance. Int. J. Parasitol. 54, 259-268.

Raupach, G.S., Kloepper, J.W. 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88, 1158ï 1164.

Sangster, N.C. 1999. Anthelminthic resistance: Past, present and future. Int. J. Parasitol. 29, 115-124.

Stirling, G.R. 1991. Biological Control of Plant Parasitic Nematodes: Progress, Problems and Prospects. CAB International, Wallingford, UK.

Van Wyk, J.A., Malan, F.S., Randles, J.L. 1997. How long before resistance makes it possible to control some field strains of *Haemonchus contortus* in South Africa with any of the anthelminthics? Vet. Parasitol. 70, 11-122.

Waller, P.J. 1994. The development of anthelmintic resistance in ruminant livestock. Acta Trop. 56, 233-243.

Waller, P.J. 2003. Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy, with emphasis on biological control. An. Health Res. Rev. 4, 35-43.

Zhao, M.L., Huang, J.S., Mo, M.H., Zhang, K.Q. 2005. A potential virulence factor involved in fungal pathogenicity serine-like protease activity of nematophagous fungus *Clonostachys rosea*. Fungal Divers. 19, 217-234.

CHAPTER SIX

EFFECT OF *BACILLUS THURINGIENSIS* (BERLINER), *CLONOSTACHYS ROSEA* (SCHROERS) AND DIATOMACEOUS EARTH ON SHEEP NEMATODES: FEEDING TRIAL

M.A. Baloyi and M.D. Laing

Discipline of Plant Pathology, School of Agricultural Sciences and Agribusiness,

University of KwaZulu-Natal, Private Bag X01, Scottsville 3201, Pietermaritzburg,

South Africa

Abstract

The ability of biocontrol agents and diatomaceous earth (DE) to reduce the number of nematode counts was evaluated in adults and lambs of Merino sheep. The sheep were naturally infected with a mixed culture of gastrointestinal nematodes, primarily *Haemonchus contortus*. A selected strain of *C. rosea* (AB8) was grown on barley grains. The spores of a selected strain of *Bt* (AB2) were grown on wheat bran. The cultures of *Bt* and *C. rosea,* together with a commercial formulation of DE were mixed with a complete diet and fed once daily to the sheep according to their individual body weights. Results indicate that there was a significant effect of treatments on body weight gain of sheep (P = 0.032). Treatment of *Bt* 1g kg^{-1} BW, *Bt* 2g kg^{-1} BW, and DE resulted in body weight gain of 34.3, 35 and 32 kg, respectively. *C. rosea* showed a lower body weight gain of 26.2 kg and was outperformed by the Control (30.7 kg). There was no significant effect of treatments on egg per gram (EPG) (P = 0.440). Treatments significantly reduced larvae per gram (LPG) counts (P < 0.001). Treatments had mean LPG counts of 55, 59, 34 and 69 for *Bt* 1g kg^{-1} BW, *Bt* 2g kg^{-1} BW*, C. rosea* and DE, respectively. Control group had a mean LPG of 423. The mortality of L3 after application of treatment were 74.6%, 75.1%, 84.6%, 68.5% and 27.5% for, *Bt* 1g kg^{-1} BW, *Bt* 2g kg^{-1} BW, *C. rosea,* DE and the Control group, respectively. Daily feeding of BCAs to sheep was effective in reducing the numbers of L3 larval stages.

6.1 Introduction

Anthelmintic resistance by gastrointestinal nematodes (GIN) to anthelmintic drugs constitutes a major threat to the productivity of small ruminants worldwide (Prichard, 1994; Waller, 1994; Van Wyk et al., 1997; Sangster, 1999; Waller, 2003; Kaplan, 2004). There is an urgent need to find alternative or complementary solutions to anthelmintic resistance (Waller, 1999). Biological control has been identified as one of the promising alternatives to anthelmintic drugs.

Biological control involves the use of natural enemies of nematodes to reduce the number of nematodes developing on pastures to a level which is sub-clinical (Larsen, 2006). *Duddingtonia flagrans* (Cooke) has been the most researched biocontrol agent (BCA) that targets free-living stages of nematodes in livestock faeces (Larsen et al., 1992; Waller and Faedo, 1993; Larsen et al., 1998; Larsen, 2000; Waller et al., 2001b; Waller et al., 2004).

There are other microorganisms that target nematodes, including fungi, bacteria, viruses, insects, mites and some invertebrates (Stirling, 1991; Siddiqui, et al., 1996). *Bacillus thuringiensis* (Berliner) has also shown the ability to destroy free-living and adult stages of sheep nematodes (Kotze et al., 2005). The mode of action has been shown to be the same as that involved in insects (Marroquin et al., 2000). *Clonostachys rosea* (Schroers) is a facultative parasite of pathogenic plant nematodes (Yang et al., 2000). The hydrolytic enzymes (protease and chitinase) produced by *C. rosea* play a key role in its ability to penetrate and kill a nematode host (Zhao et al., 2004).

Diatomaceous earth (DE) is a naturally occurring siliceous sedimentary compound derived from the microscopic remains of unicellular algae-like plants called diatoms. It contains elements such as silicon oxide (SiO_2), aluminium oxide (Al_2O_3), iron oxide (Fe_2O_3), calcium oxide (CaO) and magnesium oxide (MgO), to name a few. The mode of action of DE is mechanical. The diatom particles have sharp edges that pierce the skin of nematodes when in contact, causing the parasite to dehydrate and die.

Other researchers have demonstrated the potential of biocontrol agents to control livestock nematodes. However, much of this research has been from *in vitro* studies. Therefore, this study aimed to determine the effect of the biocontrol agents in the field (*in vivo*)by testing the effects of *B. thuringiensis, C. rosea* and DE on larvae of sheep nematodes.

6.2 Materials and Methods

6.2.1 Formulation of biocontrol agents

Isolates of *Bt* (B2(4)M and *C. rosea* (Paec 8) were previously isolated from soil of the Livestock Section of the University of KwaZulu-Natal, Pietermaritzburg.

These were grown on wheat bran and barley grain, respectively, by[1] Plant Health Products (PHP) (Pty) Ltd.

6.2.2 Animal grouping

Fourty sheep per treatment, naturally infected with mixed cultures of GIN were used in this experiment. The predominant species was *H. contortus* ([2]Allerton Veterinary Laboratory). The sheep were grouped according to age (young and adults) and by sex (ewes and rams). Sheep were kept in feeding crates in the sheep facility at Ukulinga Research Farm. The feeding crate size was 0.90 × 1.50m. These were each provided with an individual feeder and drinker. The flooring was of slatted wood to ensure that urine and faeces did not accumulate near the sheep. The facility was temperature regulated with large fans.

6.2.3 Feed preparation

The standard sheep feed consisted of the following ingredients: cottonseed cake (37.5kg), hormony chop (33.3kg), liquid molasses (8.6kg) and Vitamin Premix (1.1 kg). The *Bt* product was added to the standard feed at a rate of either 1 or 2g per kg body weight (BW) for each sheep. The *C. rosea* product was added to the standard feed at a rate of 1g per kg BW for each sheep. The DE product was added to the standard feed at a rate of 15% by weight of the BW of each sheep. The products were thoroughly mixed with the standard feed before being given to the sheep. The sheep were fed 1.5% of the mixed feed per kg of their body weight daily between 07:00-09:00am and then given hay *ad libitum*.

6.2.4 Experimental procedure

Pre-treatment period

During a pre-treatment period, the sheep were randomly allocated to individual sheep feeding stalls to acclimatise them to the handling facilities for 2 wk. Sheep were given feed supplement in the morning and hay *ad libitum*. At the end of this 2 wk period, initial values of response variables namely, body weight, EPG, LPG (larva per gram) and LD (larval development) were determined.

[1]Plant Health Products (Pty) Ltd., P.O. Box 207, Nottingham Road, South Africa

During treatment period

Eight sheep were assigned to one of the five treatments, (i) two levels of *Bt* application (1 and 2g kg^{-1} BW), (ii) *C. rosea*, (iii) 15% DE and (iv) a Control group at 1g kg^{-1} BW per day of the standard ration formulation. All the treatments were included in the total daily mixed ration formulation for 1 wk.

Post-treatment period

Treatments were stopped and sheep were fed the standard feed for a period of 1 wk.

6.2.5 Parasitology and animal performance

Sheep were weighed weekly. Rectal grab samples were collected weekly and sent to the Allerton Veterinary Laboratories (No. 458 Town Bush Road, Montrose, Pietermaritzburg, South Africa) for larval identification (LID), while faecal egg count (FEC) were calculated using the McMaster Method (Whitlock, 1949). Two grams of sheep faeces were weighed and mixed with 52ml of a saturated table salt solution. The aliquot was stirred in a N, W, S, E direction and a sample pippetted onto a McMaster slide for the counting of eggs under a light microscope (x100). Samples were counted within 15 min to avoid the eggs being distorted due to the hypertonic salt solution. Larval mortality was determined by weighing 2g samples of faeces from each sheep into a microtitre plates. The faeces were well aerated, incubated for 7d and moistened when necessary. The infective larvae were collected using the Baermann technique. Counting of larvae was performed using three aliquots of 2ml from each culture under a dissecting microscope.

6.2.6 Statistical analysis

Percent mortality of the nematodes was determined using Abbottôs Formula (Abbott, 1925), which is as follows:

$$\text{Mortality} = \frac{[C1 - C2]}{C1} \times 100$$

where: C1 = mean number of larvae in untreated manure; C2 = number of larvae in treated manure

Means were compared by Fisherôs LSD at a significance level of 5%.

Larvae per gram from each sample was obtained by dividing the total number of larvae counted in each sample of 2g of faeces used, by 2 to obtain nematode counts in 1g, as described by Ojeda-Robertos et al., (2008).

Larval development (LD) was calculated by the formula of Paraud et al. (2005), which is as follows:

$$LD = (EPG/LPG) \times 100$$

Where: EPG is the egg per gram, LPG is the larvae per gram.

ANOVA was applied to the values of BW, EPG, LPG, mortality and LD. The Genstat® statistical package was used, after transforming the EPG and mortality by log transformation to normalize homogeneity of variance of the data. The mortality of L3 nematodes was analysed using ANOVA, as a percentage of the Control L3 nematode counts, after the data was log transformed. These data are presented in the tables together with the untransformed means. The transformed data was used to calculate LSD values to separate treatment means at 5% significance level.

6.3 Results

There was a significant difference in sheep body weights as a result of the treatments (Table 6.1). Sheep fed with the two *Bt* dose treatments resulted in similar body weights which were higher than all the other treatments. *C. rosea* did not have any significant effect on the body weights of the animals.

6.3.1 Effect of treatments on body weight

Table 6.1: Effect of treatments on the body weight of sheep when treated with two levels of *Bacillus thuringiensis* (*Bt*)*, Clonostachys rosea*, and diatomaceous earth (DE) as feed additives for 1 week

Treatment	**Body weight change (kg) (Final)**
Bt 1g kg^{-1} BW	34.38**b**
Bt 2g kg^{-1} BW	35.0**b**
C. rosea	26.25**a**
DE	32.06**b**
Control	30.75**a**
F-ratio	3.08
P-value	0.032*
LSD	5.762
CV%	17.8

* Denotes significantly different at 5% probability
Means followed by the same letter are not significantly different

6.3.2 Effect of treatments on egg per gram (EPG)

There was a significant difference between nematode egg counts (Table 6.2). However, sheep fed *C. rosea* had the lowest egg counts, which suggests that *C. rosea* is active against nematode eggs, which was expected.

Table 6.2: Effect of treatments on the faecal egg counts of nematodes of sheep when treated with two levels of *Bacillus thuringiensis* (*Bt*)*, Clonostachys rosea* and diatomaceous earth (DE) as feed additives for 1 week

Treatment	**Transformed EPG (Log transformed)**	**Mean values of EPG**	**Percentage of Control**
Bt 1g kg^{-1} BW	2.902	1681	9.5
Bt 2g kg^{-1} BW	2.688	942	49.3
C. rosea	2.513	923	50.3
DE	3.039	1835	1.2
Control	2.933	1858	0
F-ratio	0.97		
P-value	0.440**NS**		
LSD	0.6219		
CV%	21.6		

NS denotes not significantly different at 5% probability

6.3.3 Effect of treatments on larvae per gram (LPG)

There was a significant difference between the treatments for the LPG counts. *C. rosea* was the best biological control agent treatment resulting in the least number of LPG counts of nematodes. All treatments were effective in reducing the nematode larval counts (Table 6.3). As a percentage of the LPG of the control, the counts for the treated sheep were in the range of 83.7% to 92%. DE was the least effective treatment with an LPG of 69 which was 83.7% of the Control.

Table 6.3: Effect of treatments on the larvae per gram of nematodes of sheep when treated with two levels of *Bacillus thuringiensis* (*Bt*)*, Clonostachys rosea* and diatomaceous earth (DE) as feed additives for 1 week

Treatments	**Transformed LPG values (Log transformed)**	**Mean LPG (final)**	**Percentage reduction in relation to Control**
Bt 1g kg^{-1} BW	1.787**a**	55	87
Bt 2g kg^{-1} BW	1.787**a**	59	86.1
C. rosea	1.622**a**	34	92
DE	1.875**a**	69	83.7
Control	2.405**b**	423	0
F-ratio	9.57		
P-value	< 0.001***		
LSD	0.2803		
CV%	14.4		

* Denotes significantly different at 5% probability
Means followed by the same letter in the same column are not significantly different

6.3.4 Effect of treatments on corrected mortality (Abbott's Correction)

The mortality of nematodes was significantly different for the four treatments applied (Table 6.4). The *Bt* doses were also very effective and caused nematode mortalities of 74.6% and 75.1% (1g kg^{-1} BW and 2g kg^{-1} BW, respectively). *C. rosea* was the best biocontrol agent resulting in a nematode mortality of 84.6%. DE ranked fourth, but was also effective, causing a nematode mortality of 68.5%.

Table 6.4: Effect of treatments on nematodes of sheep when treated with two levels of *Bacillus thuringiensis* (*Bt*), *Clonostachys rosea* and diatomaceous earth (DE) as feed additives for 1 week

Treatment	**Mortality Transformation (Log transformed)**	**Mortality (%) (final)**
Bt 1g kg^{-1} BW	1.795**b**	74.6
Bt 2g kg^{-1} BW	1.833**b**	75.1
C. rosea	1.945**b**	84.6
DE	1.746**b**	68.5
Control	1.260**a**	27.5
F-ratio	6.25	
P-value	0.001***	
LSD	0.3072	
CV%	17.5	

Mortality results were log transformed

* Denotes significantly different at P Ò0.005

6.3.5 Effect of treatments on Larval Development (LD)

None of the treatments significantly reduced LD (Table 6.5). The best treatment was *C. rosea* that resulted in the smallest LD of 2%. *Bt* at 1g kg^{-1} BW resulted in an LD of 20%. However, more larval development was observed in *Bt* at 2g kg^{-1} BW. The DE treatments resulted in an LD of 11%. However, there was more LD in the Control than in any of the treatments.

Table 6.5: Effect of treatments on the larval development of nematodes of sheep when treated with two levels of *Bacillus thuringiensis* (*Bt*)*, Clonostachys rosea* and diatomaceous earth (DE) as feed additives for 1 week

Treatment	**LD Transformation (Log transformation)**	**Larval development (%) means**	**Percentage of Control**
Bt 1g kg^{-1} BW	1.335	20.0	69.3
Bt 2g kg^{-1} BW	1.619	42.0	35.4
C. rosea	1.467	2.0	96.9
DE	1.443	11.0	83.1
Control	1.771	65.0	0
F-ratio	2.28		
P-value	0.092**NS**		
LSD	0.3293		
CV%	20.9		

Larval development was calculated by the number of larvae (LPG) divided by the egg counts (EPG) x 100

NS denotes not significantly different at 5% probability

6.4 Discussion

Body weight

There were significant differences in body weights between treatments, regardless of the fact that the trial was run for too short a period for the weight of sheep to change. Similarly, Knox and Faedo (2001) reported positive body weight gain in sheep treated with *D. flagrans*. In contrast, Hadley et al. (1987) found that there was no change in the body weights of sheep treated with *Bt*. Larsen et al. (1995), Eysker et al. (2006), and Fontenot et al. (2003) all reported that there were no differences in body weights between treated and untreated groups, in their studies on the use of *D. flagrans* to control GIN.

The sheep always consumed their feed, irrespective of the formulation of the treatment. Previous attempts of formulating biocontrol agents with *D. flagrans* included mixing spores in daily supplement, feed blocks (Waller et al., 2001b; Chandrawathani et al., 2002) and slow release devices (Waller et al., 2001a). Hence, this aspect is unlikely to have affected body weights.

Application of control agents in feed is important because they will be excreted together with the eggs in faeces. They will then germinate to parasitize the eggs and developing larvae. In this manner, pasture contamination would be reduced (Waller, 2006). Lee et al. (2002) succeeded in recovering ingested spores of *Bt* from the faeces of sheep. According to Larsen et al. (1991), for BCAs to be effective against GIN, they should withstand the passage through the gut of the alimentary tract and colonize the dung pat to prey on the free-living stages of the parasitic nematodes before these reach the L3 stages and become infective to livestock. Studies on *D. flagrans* have shown that its efficacy was proportional to its spore density (Sanyal, 2004; Sanyal et al., 2008).

Faecal egg counts (EPG)

There was no significant result because none of the agents affected adult GIN in the gut of the treated sheep. Therefore, egg laying continued unchanged. An environmentally friendly treatment that parasitizes the nematode eggs such as *Paecilomyces lilacinus* (Samson) is needed.

Meadows et al. (1989) found that toxins of *Bt kurstaki* were lethal towards eggs, and the L1, L2 and L3 larval stages of *Trichostrongylus colubriformis* (Giles). Bottjer et al. (1985) also reported the lethal effect of the toxin of *Bt israelensis* on eggs of *T. colubriformis* in the laboratory. Kotze et al. (2005) showed the ability of *Bt* Strain L366 to kill adult livestock nematodes in the laboratory. He suggested that the use of *Bt* would require the encapsulation of the *Bt* to protect it from the acidity of the abomasal lumen. Our results show that this is not necessary. Shimoda et al. (2001) and Senel and McClure (2004) proposed the use of the mucosal adhesive properties of chitosan to ensure a localisation of the *Bt* at the site of nematode infection within the mucosa or intestinal epithelium, if *Bt* is to be used as a deworming agent. According to Kotze et al. (2005), this approach would ensure a degree of protection in the neutral pH environment of secreted mucous in the microenvironment inhabited by the nematodes.

Larvae per gram (LPG)

Counts of nematode larvae were significantly different. All treatments reduced the LPG counts (P = 0.001). *C. rosea* caused lower larval counts than all the other treatments. The results parallel the mortality data.

Corrected mortality

A mortality of GIN in the livestock faeces was achieved after one week of feeding BCAs to sheep. Given that it can produce chlamydospores in culture, this fungus has considerable potential to be commercialized as a BCA against GIN. This is the first report of *C. rosea* to be used as a biocontrol agent against sheep nematodes.

There was no difference in the levels of mortality of GIN caused by the two doses of *Bt* (1g and 2g Kg^{-1} BW). *B. thuringiensis* is a widely used biocontrol agent for the control of insect pests of plants and animals. Two other sets of experiments, using *Bt* to control sheep GIN, were undertaken in the laboratory (Kotze et al., 2005; OõGrady et al., 2007). It would seem there are no other *in vivo* trials with *Bt* involving the feeding of a *Bt* product to sheep. DE also proved to be effective on nematodes, and therefore provides another treatment option.

These results accord with the successful results reported by Larsen et al., (1995) and Chandrawathani et al., (2002), on the use of the biocontrol agent *D. flagrans* to reduce sheep nematode counts in the field.

Larval development

The recovery of larvae from the faeces of treated sheep was reduced. *C. rosea* resulted in the lowest LD value of 2%. *Bt* dose of 1g kg^{-1} BW also resulted in lower counts of nematodes that developed, its LD was 20%.

In conclusion, all treatments were effective and ensured that counts of the L3 larval stages of GIN were reduced. Other researchers have shown that if the L3 population is reduced, then infection decreases and GIN count in the gut of livestock decreases and subsequently EPG is lower (Larsen, 1995; Van Wyk et al., 1999).

6.5 References

Abbott, W.S. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Ento. 18, 265-267.

Bottjer, K.P., Bone, L.W., Gill, S.S. 1985. Nematoda: susceptibility of the egg to *Bacillus thuringiensis* toxins. Exp. Parasitol. 60, 239-244.

Chandrawathani, P., Jamnah, O., Waller, P.J., Höglund, J., Larsen, M., Zahari, W.M. 2002. Nematophagous fungi as a biological control agent for nematode parasites of small ruminants in Malaysia: A special emphasis on *Duddingtonia flagrans*. Vet. Res. 33, 235-396.

Eysker, M., Bakker, N., Kooyman, F.N.J., Olde-Olthuis, S., Ploeger, H.W. 2006. Effect of biological control through the daily application of spores of *Duddingtonia flagrans* in lambs kept under an evasive grazing system in the Netherlands. Vet. Parasitol. 140, 312-320.

Fontenot, M.E., Miller, J.E., Peña, M.T., Larsen, M., Gillespie, A. 2003. Efficiency of feeding *Duddingtonia flagrans* chlamydospores to grazing ewes on reducing availability of parasitic nematode larvae on pasture. Vet. Parasitol. 118, 203-213.

Hadley, W.M., Burchiel, S.W., McDowell, T.D., Thilsted, J.P., Hibbs, C.M., Whorston, J.A., Day, P.W., Friedman, M.B., Stoll, R.E. 1987. Five month oral (Diet) toxicity/infectivity study of *Bacillus thuringiensis* insecticides in sheep. Fund. Appl. Toxicol. 8, 236-242.

Kaplan, R.M. 2004. Drug resistance in nematodes of veterinary importance: A status report. Trends Parasitol. 20, 477-581.

Knox, M.R., Faedo, M. 2001. Biological control of field infections of nematode parasite of sheep with *Duddingtonia flagrans* and effects of spore intake on efficacy. Vet. Parasitol. 101, 155-160.

Kotze, A.C., O'Grady, J., Gough, J.M., Pearson, R., Bagnall, D.H., Akhurst, R.J. 2005. Toxicity of *Bacillus thuringiensis* to parasitic and free-living life stages of nematode parasitic of livestock. Int. Parasitol. 35, 1013-1022.

Larsen, M. 2000. Prospects for controlling animal parasitic nematodes by micro fungi. Parasitology 120, S120-S131.

Larsen, M. 2006. Biological control of nematode parasites in sheep. J. An. Sci. 84, E133-E139.

Larsen, M., Faedo, M., Waller, P.J., Hennessy, D.R. 1998. The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: Studies on *Duddingtonia flagrans*. Vet. Parasitol. 76, 121-128.

Larsen, M., Nansen, P., Gronvold, J., Wolstrup, J., Henriksen, S.A. 1992. *In vivo* passage of nematophagous fungi selected for biocontrol of parasitic nematodes in ruminants. J. Helminthol. 66, 137-141.

Larsen, M., Nansen, P., Wolstrup, J., Gronvold, J., Henriksen, S.A., Zorn, A. 1995. Biological control of trichostrongylus in calves by the fungus *Duddingtonia flagrans* fed to animals under natural grazing conditions. Vet. Parasitol. 60, 321-330.

Larsen, M., Wolstrup, J., Henriksen, S.A., Dackman, C., Gronvold, J., Nansen, P. 1991. *In vitro* stress selection of nematophagous fungi for biological control of parasitic nematodes in ruminants. Vet. Parasitol. 65, 141-193.

Lee, D.H., Machii, J., Ohba, M. 2002. High frequency of *Bacillus thuringiensis* in faeces of herbivorous animals maintained in a zoological garden in Japan. Appl. Ento. Zoo. 37, 509-516.

McPartland, J. 2000. Biological control of hemp. Third In. Symposium: Bioresource hemp. (Wolfsburg, Germany).

Meadows, J., Gill, S.S., Bone, L.W. 1989. Factors influencing lethality of *Bacillus thuringiensis* kurstaki toxin for eggs and larvae of *Trichostrongylus colubriformis* (Nematoda). J. Parasitol. 75, 191-194.

O'Grady, J., Akhurst, R.J., Kotze, A.C. 2007. The requirement for early exposure of *Haemonchus contortus* larvae to *Bacillus thuringiensis* for effective inhibition of larval development. Vet. Parasitol. 150, 97-103.

Ojeda-Robertos, N.F., Torres-Acosta, J.F., Aguilar-Caballero, A.J., Ayala-Burgos, A., Cob-Galera, L.A., Sandoval-Castro, C.A., Barrientos-Medina, R.C., Mendozade Gives, P. 2008. Assessing the efficacy of *Duddingtonia flagrans* chlamydospores per gram of faeces to control *Haemonchus contortus* larvae. Vet. Parasitol. 158, 329ï 335.

Paraud, C., Hoste, H., Lefrileux, Y., Pommaret, A., Paolini, V., Pors, I., Chartier, C. 2005. Administration of *Duddingtonia flagrans* chlamydospores to goats to control gastrointestinal nematodes: dose trials. Vet. Res. 36, 157-166.

Prichard, R.K. 1994. Anthelmintic resistance. Int. J. Parasitol. 54, 259-268.

Sangster, N.C. 1999. Anthelmintic resistance: Past, present and future. Int. J. Parasitol. 29, 115-124.

Sanyal, P.K. 2004. Density dependent nematophagous behaviour of *Duddingtonia flagrans*. Indian Vet. J. 81, 16-19.

Sanyal, P.K., Patel, N.K., Mandal, S.C., Pal, S. 2008. Formulation of a strategy for the application *Duddingtonia flagrans* to control caprine parasitic gastroenteritis. J. Helminthol. 82, 169-174.

Senel, S., McClure, S.J. 2004. Potential applications of chitosan in veterinary medicine. Adv. Drug Deliv. Rev. 56, 1467ï 1480.

Shimoda, J., Onishi, H., Machida, Y. 2001. Bioadhesive characteristics of chitosan microspheres to the mucosa of rat small intestine. Drug Dev. Ind. Pharm. 27, 567ï 576.

Siddiqui, Z.A., Qureshi, S.A., Sultana, V., Ehteshamul-Haque, S., Ghaffar, A. 2000. Biological control of root-knot nematode disease complex of tomato. Plant Soil 227, 163-169.

Stirling, G.R. 1991. Biological Control of Plant Parasitic Nematodes: Progress, Problems and Prospects. CABI, Wallingford, UK.

Van Wyk, J.A., Malan, F.S., Randles, J.L. 1997. How long before resistance makes it impossible to control some field strains of *Haemonchus contortus* in South Africa with any of the anthelmintics? Vet. Parasitol. 70, 11-122.

Waller, P.J. 1994. The development of anthelmintic resistance in ruminants livestock. Acta Trop. 56, 233-243.

Waller, P.J. 1999. International approaches to the concept of integrated control of nematode parasites of livestock. Int. J. Parasitol. 29, 155ï 164.

Waller, P.J. 2003. Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy, with emphasis on biological control. An. Health Res. Rev. 4, 35-43.

Waller, P.J. 2006. Sustainable nematode parasite control strategies for ruminant livestock by grazing management and biological control. An. Feed Sci. 126, 277-289.

Waller, P.J., Faedo, M. 1993. The potential of nematophagous fungi to control the free-living stages of nematode parasites: *In vitro* and in vivo *studies*. Vet. Parasitol. 51, 289-299.

Waller, P.J., Faedo, M., Ellis, K. 2001a. The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: Towards the development of a fungal controlled release device. Vet. Parasitol. 102, 299-308.

Waller, P.J., Knox, M.R., Faedo, M. 2001b. The potential of nematophagous fungi to control the free-living stages of sheep: Feeding and block studies with *Duddingtonia flagrans*. Vet. Parasitol. 102, 321-330.

Waller, P.J., Schwan, O., Ljungstrom, B.L., Rydzik, A., Yeates, G.W. 2004. Evaluation of biological control of sheep parasites using *Duddingtonia flagrans* under commercial farming conditions on the island of Gotland, Sweden. Vet. Parasitol. 126, 299ï 315.

Whitlock, H.R. 1948. Some modifications of the Modified McMaster helminth egg counting technique apparatus. J. Council Sci. Indus. Res. 21, 177-180.

Yang, X.J., He, Y.X., Chen, F.R., Zheng, L. 2000. Separating and selection of egg-mass fungi of *Meloidogyne* sp. in Fujian province. Fujian J. of Agric. Sci. 15, 12-15.

Zhao, M.L., Mo, M.H., Zhang, K.Q. 2004. Characterization of a neutral serine protease and its full-length cDNA from the nematode-trapping fungus *Arthrobotrys oligospora*. Mycologia 96, 16-22.

THESIS OVERVIEW

The bacterium *Bacillus thuringiensis* (*Bt*) (Berliner) and the fungus *Clonostachys rosea* (Schroers) have been widely studied as biocontrol agents in pest control of plants. To date, there is still no commercialized *Bt* or *C. rosea* formulation for the control of livestock pests.

The problem of anthelmintic resistance has been the driving force behind the search for alternative control measures. Moreover, chemical residues in foods and the demand to reduce chemical inputs in agriculture are another major concern (De and Sanyal, 2009).

Biological control is becoming an important non-chemical option because biocontrol agents have low mammalian toxicity, high efficacy and are naturally occurring (Moore et al., 2000).

In the research reported in this thesis, 25 *Bt* and 10 *C. rosea* isolates were obtained from grazing pastures and pens of sheep at the Livestock Section, Ukulinga Research Farm, UKZN, Pietermaritzburg. The isolates were characterized according to their morphology. The *Bt* colonies were white in colour, had a rough texture with flat elevation, undulate margins and irregular shape. The spores were rod-shaped, stained gram positive and clear. *C. rosea* were identified by whitish colonies with the characteristic conidiophores which were branched and showed phialides at the tips. The goat grazing pasture soil was a good source for the best and most effective isolates used as biological control agents during this study.

The isolates were screened in the laboratory, and some were highly pathogenic to livestock nematode larvae. Fifteen *Bt* isolates and eight *C. rosea* isolates were effective in reducing the L3 larval stage in faeces and water bioassays.

The most effective concentration of each isolate was also determined, and indeed lower concentrations of *C. rosea* were effective in reducing larvae counts under laboratory conditions. The best concentrations were 10^{10} spores ml^{-1} and 10^{9} conidia ml^{-1} for *Bt* and *C. rosea*, respectively. *C. rosea* efficacy did not increase with the increase in concentrations tested and was the most effective biocontrol agent.

Theoretically, combinations of biocontrol agents might be more effective than a single biocontrol agent. Some *Bt* isolates improved their efficacy when combined with others and showed an

additive interaction effect. Isolate P3 of *C. rosea* was antagonistic when mixed with other bacterial isolates (B2, B10 and B12) and fungal Isolate P8. However, the level of control by this isolate was highest when used alone.

The incorporation of biocontrol agents in animal feed caused a mortality of L3 larval stages of nematodes. There was no dose response of nematode mortality to *Bt* treatment. *C. rosea* caused the greatest mortality of nematodes. Diatomaceous earth was effective against nematodes larvae. The results showed that these biocontrol agents were effective in controlling animal nematodes and should be adopted into integrated pest management programmes.

Future studies

As biocontrol agents have proven to be effective in the laboratory, field trials that extend for longer periods need to be undertaken. Further studies are needed to evaluate the efficacy in pasture, to determine whether pasture contamination is reduced.

The speed of kill of BCAs in the field under extreme environmental conditions needs to be determined. In addition, the effect of these BCAs on eggs needs to be determined.

The mechanisms of *C. rosea* involved in killing livestock nematode needs to be determined, to find out as to whether it uses the same mechanism as that used on plant nematodes.

The relative cost of daily feeding of biocontrol agents needs to be addressed relative to the cost of anthelmintics used locally.

The effects of anthelmintics such as benzimidazoles on biocontrol agents need to be studied as to whether they inhibit *C. rosea* and other nematophagous fungi. Also, common antibiotics and other medicines fed to livestock need to be tested for compatibility.

The best biocontrol agent should be tested on various livestock endoparasites to determine if they can be used as broad-spectrum control agents.

References

De, S., Sanyal, P.K. 2009. Biological control of helminth parasites by predatory fungi. Vetscan 4, 1-9.

Moore, D., Lord, J.C. Smith, S.M. 2000. Pathogens. In: Subramanyam, B., Hagstrum, D.W., (Eds.), Alternatives to Pesticides in Stored-Product IPM, Kluwer Academic Publishers, Dordreecht, pp. 193ï 227.

APPENDIX

Appendix 1: Characterization of *Bacillus thuringiensis* isolates

	Morphological characteristics of colony					Spores				
Isolate	colour	Texture	Elevation	Margin	Shape	Shape	Gram stain	Shape	colour	Position
B1(1)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B1(2)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B1(3)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B1(4)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B2(1)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B2(2)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B3(3)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B4(1)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B4(2)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B3(4)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B1(4)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B3(6)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B5(3)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B6(3)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B6(2)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B5(1)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B7(2)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B8(3)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B10(4)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B10(2)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B10(3)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B9(2)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B9(4)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B10(1)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B9(3)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B8(2)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre

B5(2)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre	
B10(5)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre	
B11(1)	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre	
B12	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre	
B13	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre	
B14	Bream wrhite	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre
B15	white	white	Rough	Flat	Undulate	Irregular	Rod	+	Oval	Clear	Centre