African Journal of Agricultural Research Vol. 6(22), pp. 5047-5054, 12 October, 2011
Available online at http://www.academicjournals.org/AJAR
DOI: 10.5897/AJAR11.261
ISSN 1991-637X ©2011 Academic Journals

Full Length Research Paper

Isolation and *in vitro* screening of *Bacillus thuringiensis* and *Clonostachys rosea* as biological control agents against sheep nematodes

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

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

Accepted 9 August, 2011

Biological control agents are possibly the best alternative to agrochemicals for the control of livestock nematodes. Strains of *Bacillus thuringiensis (Bt)* and *Clonostachys rosea* were isolated from grazing pastures collected from pens of livestock. A total of 25 isolates of *B. thuringiensis* and 10 of *C. rosea* were successfully isolated. *In vitro* studies were conducted to determine the efficacy of *B. thuringiensis* (*Bt*) and *C. rosea* isolates as biological control agents against sheep nematodes. All of *Bt* and *C. rosea* reduced nematode counts considerably. Isolates of *Bt* and *C. rosea* reduced nematodes counts by 28.5 to 62% and 44 to 69.9%, respectively, in the faeces 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. Majority of the best nematode-killing isolates were from a goat grazing pasture. Both biocontrol agents showed capability to reduce nematode counts in this study.

Key words: Anthelmintics drugs, *Bacillus thuringiensis*, biological control agents, *Clonostachys rosea*, livestock nematodes.

INTRODUCTION

Gastrointestinal parasite infection is the most important limiting factor to sheep production worldwide, resulting in serious economic losses (Perry and Randolph, 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.

Gray (1993) classified the principal livestock nematodes according to their location in the host, which included those that attack the abomasums: *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). 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). *H. contortus* is the most important

*Corresponding author. E-mail: YoboK@ukzn.ac.za. Tel: (0027)332605822. Fax: (0027)332606475.

nematode species of sheep in tropical and subtropical areas, or regions with summer-dominant rainfall. Infested sheep may be seen by severe weight loss and anaemia and pale gums and on the insides of the eyelids. Bottle-jaw may also occur at more than 10 days post-infection (O'Conner et al., 2006). *H. contortus* becomes less significant as the climate tends towards winter (O'Connor et al., 2006).

Bacillus thuringiensis (*Bt*) (Berliner) is a heterogeneous species of rod-shaped, gram-positive bacterium, which produces Cry toxins proteins during sporulation. These are used in insect pest control in agriculture (Schnepf, 1998). However, some *Bt* strains also show activity against stock nematodes (Kotze et al., 2005). Kotze et al. (2005) demonstrated that isolates of this bacterium were able to consistently reduce larval counts and adult populations of these nematodes. In addition, Kotze et al. (2005) 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. 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, 1998; Siegel, 2001). Lee and Pankhurst (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).

The fungus *Clonostachys rosea* (Schroers) has mostly been used as a biological control agent on plant parasitic nematodes (Li et al., 2006). This fungus is commonly found in soil (Sutton, 1997). According to Zhao et al. (2005) and Li et al. (2006), this fungus destroys nematodes due its ability to penetrate cuticle through production of a germ tube which penetrate the host and kills it. This has raised the possibility of using this nematophagous fungus to destroy gastrointestinal nematodes (GIN) of sheep. Consequently this study aimed to isolate these two biocontrol agents, *Bt* and *C. rosea* and to evaluate their pathogenicity on the larvae of a mixed culture of sheep nematodes in *in vitro* trials, conducted in both sheep faeces and in water.

MATERIALS AND METHODS

Sample collection

Samples were collected from different sites in the livestock section at the Ukulinga research farm (30° 24'S, 29° 24'E at an altitude of 700m), University of KwaZulu-Natal, Pietermaritzburg, South Africa. The samples comprised of soil from pens of cattle, sheep and goats, and soil from the grazing pastures of horses, sheep, cattle and goats. Surface soil (15 mm) was removed with a spatula and approximately 20 g soil samples were collected into a plastic bag. Soil samples were stored in a fridge at 4°C until used.

Isolation of *Bacillus thuringiensis*

B. 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 9 ml of sterile distilled water and heat treated for 15 min at 80°C in a rotary water bath. The suspensions were serially diluted, plated onto nutrient agar and kept at room temperature for 4 d to sporulate, then harvested into tryptone soy broth, shaken at 150 rpm for 1wk at 28°C, then centrifuged (10,000 rpm, 10 min, 4°C), once in 0.01M NaCl, and twice in cold sterile distilled water. The final pellet was re-suspended in 4 ml of sterile distilled water and stored at -80°C (as described by Kotze et al., 2005).

Isolation of *Clonostachys rosea*

A basal medium containing of $MgS0_4$(0.1 g), K_2HPO_4(0.4g), KCl (0.075 g), NH_4NO_3 (0.5 g), glucose (1.5 g) and agar (10 g) and crystallized chloramphenicol (0.25 g) were mixed and added to 475 ml distilled water and autoclaved. Propamocarb (previcur) fungicide (0.6 g) was added to 50 ml of distilled water and separately added to the basal medium. A one gram sample of soil was suspended in 9 ml of sterile distilled water and plated on the medium. The plates were incubated at 25°C and monitored for the development of lilac colonies. These colonies were subcultured onto PDA and incubated at 25°C, for 1 wk.

Identification of isolates

To look for presence of parasporal bodies of *Bt*, an aliquot of the 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% Coomasie Blue stain in 50% acetic acid), rinsed with distilled water, dried and observed with a light microscope (× 1000) under oil immersion (Ammons and Ramphersad, 2002). Mycelium was taken from 2 wk old cultures of *C. rosea* grown on PDA and mounted on a slide and stained with lactophenol cotton blue. The identity of the *C. rosea* cultures was confirmed by the mycology section of the plant protection research institute, agricultural research council (PPRI-ARC, 600 Soutpansberg Road, Ritondale, Pretoria, South Africa) using morphological traits and DNA sequencing.

Pathogenicity bioassays

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 infected merino sheep that fed on grass pastures. The sheep had a mixed infection of nematodes (natural infections) which was dominated by *Haemonchus contortus*. 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 microtiter plate, then treated with a *Bt* cell suspension (10^8 spores/ml) or a *C. rosea* conidial suspension (10^6 spores/ml) and the control group was treated with distilled water, 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 facilitate air flow. Plates were incubated at 25°C for 7 d and nematode larvae were recovered using the baermann technique (Ichhpujani and Bhatia, 1998).

Table 1. Sources and distribution of *Bacillus thuringiensis* isolates from soil.

Sample habitat	Number of soil samples examined	Total samples with *Bt*	Number of coloniese xamined	Number of *Bt* colonies	*Bt* %
Sheep pen soil	5	3	86	5	5.8
Goat pasture soil	5	3	112	6	5.4
Cattle pen soil	5	2	97	4	4.1
Sheep pastures	5	3	73	5	6.9
Goat pen soil	5	4	90	5	5.6
Cattle kraal soil	5	-	87	-	0
Horse pasture soil	5	-	76	-	0
Total	35	15	458	25	5.45

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

Bioassay in water

Faeces were collected directly from the rectum of sheep infected with a mixed culture of nematodes. The faeces were incubated for 4d at 25°C. 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°C for a minimum of 8 h. The nematodes were surface sterilized in sodium hypochlorite (1% v/v) for 10 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 to 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 (Grady et al., 2007) and the samples were incubated at 25°C. After incubation, the numbers of nematode larvae were counted under a dissecting microscope.

Statistical analysis

Treatments were arranged in a randomized complete blocks design with three replications. The experiment was repeated three times for the faecal culture bioassay and two times for water bioassay. The nematode count values were pooled. The nematode counts of the faeces and water bioassays were angular transformed to normalize the data. ANOVA was applied to the data. If the F-test was significant, then the means of nematode counts were compared using Duncan's multiple range test or Fisher's least significant difference test (LSD). 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:

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

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

RESULTS

Isolation and identification of *B. thuringiensis and C. rosea*

Bacillus thuringiensis was isolated from 15 out of 35 (43%) soil samples (Table 1). The number of *Bt* isolates found was similar in soil samples from sheep pens, a sheep grazing pasture and a goat grazing pasture, with each generating 20% of *Bt* samples. The majority of samples with *Bt* isolates were found in the soil of a goat pen (27%). The soil of a cattle pen had fewer *Bt* isolates (13%), whereas the soil of a cattle kraal and horse pasture had no *Bt*. Amongst the 458 bacterial colonies examined, 25 of them were identified as *Bt* isolates, resulting in a mean *Bt* index of 0.05. (Table 1) The colony characteristics of *Bt* isolates were circular, white, flat and undulate. Thirty out of 458 bacteria isolated were gram-positive and rod-shaped. Twenty-two percent 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, 83.3% of the gram-positive bacterial isolates were *Bacillus* spp. Colonies of *C. rosea* were velvety in texture.

The colours of colonies were initially white, and then became yellow in some isolates. Hyaline septate hyphae and defined branching patterns were observed in the isolates. Conidiophores were straight, with conidia produced at the tip, which were rod- shaped.

Table 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	43.3cdefg
Bt 1(2)M	55.2	48.0efg
Bt 1(4)M	41.0	39.7bcd
Bt 10(2)M	60.5	51.1fg
Bt 10(3)M	49.5	44.7defg
Bt 10(4)M	54.8	47.9efg
Bt 10(5)M	44.0	41.5bcdef
Bt 11(1)M	55.2	48.1efg
Bt 12M	57.0	49.1efg
Bt 13M	45.8	42.4bcdefg
Bt 14M	53.0	46.6efg
Bt 15M	52.0	46.2efg
Bt 2(4)M	64.0	53.2g
Bt 3(4)	31.0	33.5bc
Bt 4(1)M	56.0	48.5efg
Bt 4(2)M	43.0	41.0bcde
Bt 5(2)M	42.0	40.4bcde
Bt 5(3)M	49.5	44.7defg
Bt 6(2)M	58.0	49.6efg
Bt 6(3)M	50.2	45.2efg
Bt 7(2)M	28.5	33.2bc
Bt 8(3)M	45.2	42.2bcdefg
Bt 9(2)M	56.2	48.7efg
Bt 9(4)M	50.8	45.4efg
*Bt*10(1)M	62.5	52.3fg
Ctrl	0.0	00.0a
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 Duncan's multiple range test at a 5% level.

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

The different isolates of *Bt* were significantly different in their activity against livestock nematodes (Table 2) when compared to the control. Similarly, different isolates of *C. rosea* were significantly different in their ability to reduce the number of infective larvae (P <0.001) (Table 3).

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%. Three isolates, *Bt*2(4)M, *Bt*10(1)M, *Bt*10(2)M, caused a nematode mortality of more than 60% in faeces. Eleven *Bt* isolates caused mortality of less than 50%. Isolate *Bt*7(2)M caused the least control of nematodes, whereas *Bt*2(4)M was the most effective *Bt* isolate. Two isolates of *Bt* killed less than 31% of the nematodes. Roughly one third of the *Bt* isolates controlled nematodes by 41% to 49.5%. Eleven of the isolates caused nematode mortality in the range of 50% to 58%, with four isolates providing control levels greater than 60%.

Some isolates of *C. rosea* were effective in reducing nematode populations in faeces by more than 60%. The best isolate caused a mortality of 69.9% (Table 3). Eight of the 10 *C. rosea* isolates killed more than 50% of the nematodes in faoeces. Isolates Paec1, Paec3 and Paec8 were the best three isolates, causing mortalities of 67.6, 68.3 and 69.9%, respectively.

Pathogenicity of isolates in a water bioassay

The best three isolates of *Bt* significantly reduced nematode numbers (P<0.001). However, there was no

Table 3. Mean mortality (%) of gastrointestinal nematodes (L3 stage) 7 d, after exposure to treatment with C. rosea isolates at a concentration 106 conidia ml-1 in a sheep faeces bioassay.

Fungal isolates	Mean mortality (%)	Transformed mean
Paec8	69.9	56.9c
Paec3	68.3	55.8c
Paec1	67.9	55.5c
Paec4	65.5	54.1bc
Paec2	65.0	53.8bc
Paec5	60.5	53.3bc
Paec6	61.6	51.8bc
MM	51.5	45.9bc
B-40	48.9	44.5bc
P7	46.3	42.2b
Ctrl	0.0	0.0a
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; Means were transformed using the angular transformation; F-ratio and P-value after angular transformation; *values are means of a three replicate experiment repeated two times.

difference between the isolates in their ability to kill nematodes *in vitro* in the water bioassay, causing mortality levels of 62.0, and 85.0% (Isolates *Bt*2 (4)M, *Bt*10M and *Bt*12M, respectively) (Table 4). The best three *C. rosea* isolates significantly reduced the nematode counts in the Water Trial (P<.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 5).

DISCUSSION

Isolation

This study showed that the soils of the grazing pastures of livestock are rich in *Bt.* This confirms the findings of Martin et al. (1989) and Attathom et al. (1995), who reported that *Bt* can be isolated from most soils, including desert, beach and tundra soils. Moreover, it has also been isolated from dung of sheep, cattle, goat, deer, horses, and rabbits; soil from grazing pastures; silage and garden compost (Zhang et al., 2000). Das et al. (2008) noted that *Bt* 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 et al. (2000). Goat pen soil and sheep grazing pasture soil gave similar numbers of isolates of *Bt*. Soil from cattle grazing pasture produced only three isolates, while there were no *Bt* isolates sourced from soils of a cattle kraal and a 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 on the value of using stained specimens and light microscopy instead of phase-contrast microscopy, and described parasporal bodies such as those observed.

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 St. Germain and Summerbell (1996). Hyaline septate hyphae with defined branching patterns resembling that of *C. rosea* were observed. Macroscopic characteristics of the reverse of the colonies were dirty white and brownish in all the isolates (de Hoog et al., 2000). *Clonostachys rosea* has also been isolated from cultivated soils, forests, grassland and woodlands, heathland, freshwater and coastal soil (Gan et al., 2007).

Screening of biocontrol agents

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).

Table 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°C in a water bioassay.

Isolate	Untransformed mortality (%)	Transformed mortality (%)
B2	62.4	0.0850b
B10 (2)	62.0	0.0917b
B12	85.0	0.0937b
Ctrl	0.0	0.0a
F-ratio		181.96
P-value		<0.001
LSD		0.01160
CV %		8.6

Means are the square-root arcsine transformed; F-ratio and P-values after square root-arcsine transformation; *values are means of a three replicate experiment repeated three times.

Table 5. Mortality of nematodes larvae treated with *Clonostachys rosea* conidial suspensions at a concentration of 10^6 conidia ml^{-1} for 7 d at 25°C in a water bioassay.

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

Means followed by the same letter are not significantly different; *values are means of three replicate experiment repeated two times.

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 et al. (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). We have now 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, whereas soil from a goat grazing pasture and goat pen 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 Larsen's (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. However, higher mortalities were observed in the water bioassay than in the faeces bioassay. A number of factors might have contributed to such this, including the unequal numbers of initial nematodes present in the faeces, thus causing possible errors in the final nematode counts. A careful and proper standardization of nematode numbers may reduce such errors to a minimum and hence allowing for proper treatment comparisons.

Sheep are mostly infected with a mixed culture of nematodes, thus an effective biocontrol agents 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. Some research trials demonstrated that *Duddingtonia flagrans* was effective in reducing nematode larvae (Larsen et al., 1998). 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.

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. Entomol. Res., 85: 167-173.

Borgsteede FHM (1998). Gastrointestinal helminthiasis: Anthelmintic resistance and how to prevent and control. Symp. Parasitol. Int., 47: 23-48.

Chandrawathani P, Jamnah O, Waller PJ, Larsen M, Gillespie AT, Zahari WM (2003). Biological control of nematode parasites of small ruminants in Malaysia using the nematophagous fungus *Duddingtonia flagrans.* Vet. Parasitol., 117: 173-183.

Chatterjee SN, Bhattacharya T, Danger TK, Chandra G (2007). Ecology and diversity of *Bacillus thuringiensis* in soil environment. Afr. J. Biotechnol., 6: 1587-1591.

Coles GC (2005). Anthelmintic resistance—looking to the future: a UK perspective. Res. Vet. Sci. 78: 99-108.

Das J, Das B, Dangar TK (2008). Microbial populations and *Bacillus thuringiensis* diversity in saline rice field soils of coastal Orissa, India. Afr. J. Microbiol. Res., 2: 326-331.

De Hoog GS, Guarro J, Figueras MJ, Gené J (2000). Atlas of Clinical Fungi. 2nd ed. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands and Universitat Rovira i Virgili, Reus, Spain, p. 1124.

De S, Sanyal PK (2009). Biological control of helminth parasites by predatory fungi. Vetscan, 4: 1-8.

Faedo M, Krecek RC (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. Environ. Microbiol., 64: 4246-4254.

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. Microbiol., 45: 422-430.

Georgi JR (1969). Parasitology for Veterinarians. W.B. Saunders Company. London.

Ghahfarokhi MS, Abyaneh MR, Bahadori SR, 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.

Glare TR, O-Callaghan M (2000). *Bacillus thuringiensis*: Biology, Ecology and Safety. Wiley, Chichester.

Grady J, Akhurst RJ, Kotze AC (2007). The requirement for early exposure of *Haemonchus contortus* larvae to *Bacillus thuringiensis* for effective inhibition of larval development. Vet. Parasitol., 150: 97-103.

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

Hastowo S, Lay BW, Ohba M (1992). Naturally occuring *Bacillus thuringiensis* in Indonesia. J. Appl. Bact., 73: 108-113.

Henderson CF, Tilton EW (1955). Tests with acaricides against the brown wheat mite. J. Econ. Ento., 48: 157-161.

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

Jackson F, Coop RL (2000). The development of anthelmintic resistance in sheep nematodes. Parasitology, 120: S95–S107.

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

Kotze AC, O'Grady J, Gough JM, Pearson R, Bagnall DH, Akhurst RJ (2005). Toxicity of *Bacillus thuringiensis* to parasitic and free-living life stages of nematode parasitic of livestock. Int. Parasitol., 35: 1013-1022.

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

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

Larsen M, Faedo M, Waller PJ (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 Aust. J. Vet. Parasitol., 53: 275-281.

Larsen M, Faedo M, Waller PJ, Hennessy DR (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 KE, Pankhurst CE (1992). Soil organisms and sustainable productivity. Austr. J. Soil Res., 30: 855-892.

Li J, Yang JK, Huang XW, Zhang KQ (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 PAW, Travers RS (1989). Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol., 55: 2437-2442.

O`Connor LW, Stephen W, Brown W (2006). Ecology of the free-living stages of major Trichostrongyloid parasites of sheep. Vet. Parasitol., 142: 1-15.

Ohba M, Aizawa K (1986). Distribution of *Bacillus thuringiensis* in soils of Japan. J. Invert. Pathol., 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.

Perry BD, Randolph TF (1999). Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. Vet. Parasitol., 84: 145-168.

Perry BD, Randolph TF, McDermott JJ, Sones KR, Thornton PK (2002). Investing in Animal Health Research to Alleviate Poverty. International Livestock Research Institute (ILRI), Nairobi, Kenya, p. 148.

Sangster NC (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, Zeigler DR, Dean DH (1998). *Bacillus thuringiensis* and its pesticidalcrystal proteins. Microbiol. Mol. Biol. Rev., 62: 775-806.

Siegel JP (2001). The mammalian safety of *Bacillus thuringiensis*-based

insecticides. J. Invert. Pathol., 77: 13-21.
St. Germain G, Summerbell R (1996). Identifying Filamentous Fungi – A Clinical Laboratory Handbook, 1st ed. Star Publishing Company. California.
Sutton JC, Li DW, 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 JA, Stenson MO, Van der Merwe JS, Vorster RJ, Viljoen PG (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 AF, Letty BA, Van der Linde MJ, Van Wijk EF, Hansen JW, Krecek RC (2001). Testing for clinical anaemia caused by *Haemonchus* spp. in goats farmed under resource –poor conditions in South Africa using an eye colour chart developed for sheep. Vet. Parasitol., 99: 1-9.
Waghorn TS, Leathwick DM, Chen LY, Skipp RA (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 PJ (2003). Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy with emphasis on biological control. Anim. Health Res. Rev., 4: 35-43.
Waller PJ (2006). Sustainable nematode parasite control strategies for ruminant livestock by grazing management and biological control. Anim. Feed. Sci., 126: 277-289.
Waller PJ, Knox MR, Fardo 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 PJ, Thamsborg SM, Larsen M, Knox MR, Peter R, Miller JE, Molento MB, Beriajaya Hood GM (2004). In: Miller JE, Waller PJ (eds.) Novel Approaches to Control of Parasites-A Workshop. Vet. Parasitol., 125: 59-68.
Wei JZ, Hale K, Carta L, Platzer E, Wong C, Fang SC, Aroian RV (2003). *Bacillus thuringiensis* crystal proteins that target nematodes. Natl. Acad. Sci. USA, 100: 2700-2765.
Zhang HY, Yu ZN, Deng WX (2000). Isolation, distribution and toxicity of *Bacillus thuringiensis* from warehouses in China. Crop Prot., 19: 449-454.
Zhao ML, Huang JS, Mo MH, Zhang KQ (2005). A potential virulence factor involved in fungal pathogenicity serine-like protease activity of nematophagous fungus *Clonostachys rosea*. Fungal Divers., 19: 217-234.