Assessment of the identity, distribution and control options for seed- and leaf-gall nematodes in grass in South Africa

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A dissertation submitted in partial fulfilment of the requirements for the degree *Magister Scientiae* in Zoology at the Potchefstroom Campus of the North-West University

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November 2008

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ACKNOWLEDMENTS

I would like to express my sincere appreciation to the following people and Institute:

ARC-Grain Crops Institute for funding my studies.

My promoter, Dr. H. Fourie for giving me the chance to obtain my master's degree. I would like to thank her also for her scientific expertise, professional leadership and support. You are the best!!!

Ms. E. Venter, Ms. R. Jantjies, Ms. B. Matuli, Ms. L. Bronkhorst, Mr. A. Tladi and Mr. S. Kwena for their technical assistance throughout this study.

Professor A.H. Mc Donald for editing and reviewing the dissertation.

Dr. C. Mienie, Shaun Berry and Dr. S. Subbotin for their assistance on molecular identification and data interpretation.

Dr. A. Swart for her assistance in morphological identification of nematode samples.

Advance Seed for applying the grass seed I needed to do this study.

My father and mother for their support and love throughout the past two years. Thank you so much for everything you've done for me. I do love you so much!!!

My fiancé for his love, support and guidance the past two years, I know it wasn't always easy.

My Heavenly Farther, who created me and His continues guidance throughout my life.

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ABSTRACT

The presence of seed- and leaf-gall nematodes (SLGN; *Subanguina* spp.) in seed of *Eragrostis* spp. grass is currently a matter of great economic concern to the local grass seed industry. The adverse effect of these parasites on export as well as local markets since 2001 necessitated i) a survey in the *Eragrostis* spp. production areas of South Africa and ii) an investigation of control strategies for this parasite. *Eragrostis* spp. seed samples were obtained from 13 localities and soaked in tap water for 48 hours at 25° C for nematode extraction. The extent of SLGN infestation was quantified by calculating population density, frequency of occurrence (%) and prominence values (PV). For identification purposes, DNA fragments from these parasites were sequenced using the forward primer TW81 and reverse primer AB28. Separation of galled seeds from uninfected seeds was done in two separate trials by subjecting SLGN-infected seed to i) sieving using 250-µm-, 500-µm- and 600-µm- aperture mesh sieves and ii) flotation and sedimentation using eight NaCl concentrations, viz. 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5% and 20%. In addition the efficacy of various nematicides was evaluated under semi-controlled microplot conditions against SLGN-infected *E. curvula* (Ermelo variety) seed. SLGN from 12 of the 13 localities were identified as *S. wevelli*, with population densities and PV both ranging from 0.1 to 205 in 1g seed samples. Phylogenetic tree data indicated that these 12 SLGN populations had bootstrap values of less than 90% and did not differ significantly from each other nor from the *S. wevelli* reference population (USA). Low similarity values obtained between the ITS regions of these populations and the reference population substantiated their identification further. *Subanguina wevelli* populations from all the localities are, however, reared *in vivo* on *E. curvula* (Ermelo variety) in order to obtain mature specimens for confirmation of molecular identification. In terms of sieving, the number of *S. wevelli* contained in seed did not differ significantly for the three sieves. All three sieves did, however, contain significantly fewer *S. wevelli* than the unsieved control treatment. The seed on the 250-µm-mesh sieve had the cleanest seed (13% infested), followed by the 500-µm-mesh sieve (28% infested) and the 600-µm-mesh sieve (31% infested). Flotation-

separation, using a NaCl technique resulted in treatments not differing significantly from the untreated control (tap water only) in terms of *S. wevelli* numbers. *Subanguina wevelli* numbers in floating seed ranged from 702 to 1 952 for the 7.5% and 20% NaCl treatments, respectively, and from 84 to 392 for the 12.5% and 5% treatments in sunken seed, respectively. Combination of sieving and a 15% NaCl solution still resulted in high numbers of *S. wevelli* in seed batches. Planting of such seed will therefore still lead to *S. wevelli* infection in Eragrostis grass. Treatment of *E. curvula* seed with 0.25mg avermectin per seed, carbofuran (1.5g/m) and terbufos (0.66g/m) resulted in the greatest reduction of *S. wevelli* numbers per 2g seed and these differed significantly from the untreated control. Aldicarb, cadusafos, ethylene dibromide, ethoprophos and oxamyl treatments still had relatively high numbers of *S. wevelli* and did not differ significantly from the untreated control. Results from this study indicate that a single control strategy is not adequate to ensure that grass-seed consignments are free of SLGN. More than one control strategy, included as components of an integrated pest management system has to be applied to address this nematode problem.

OPSOMMING

Die teenwoordigheid van saad- en galaalwurms in saad van *Eragrostis* spp. gras is huidiglik van ekonomiese belang vir die Suid-Afrikaanse grassaad-industrie. Saad- en galaalwurms het sedert 2001 'n negatiewe effek op die uivoer van Eragrostis saad en gevolglik op die plaaslike mark gehad. Hierdie probleem het rolspelers in hierdie industrie genoodsaak om die volgende stappe te neem, naamlik i) 'n opname in plaaslike Eragrostis produksie gebiede en ii) metodes te laat ondersoek wat moontlik die voorkoms van hierdie aalwurm in grassaad kan beperk of heeltemal verhoed. Eragrostis saad is vervolgens deur personeel van die Landbounavorsingsraad se Instituut vir Graangewasse vanaf 13 lokaliteite ontvang wat geleë is in hierdie produksie gebiede. Een-gram saadmonsters vanaf elke lokaliteit is vir 48 uur by 25°C in kraanwater geweek om aalwurms te ekstraheer. Verder is die omvang van die saad- en gal-aalwurmbesmetting vasgestel deur die bevolkingsdigtheid, frekwensie van voorkoms (%) asook die prominensie waarde (PV) te bereken. Saad- en galaalwurms is geïdentifiseer deur sekwensie van DNS fragmente van die aalwurms vir elke lokalitet deur gebruik te maak van die voorwaartse primer TW81 en die omgekeerde primer AB28. Die skeiding van aalwurmbesmette saadgalle en gesonde saad is ondersoek deur van twee verskillende metodes/tegnieke gebruik te maak, naamlik i) sifting met 250-µm, 500-µm- en 600-µm grootte siwwe en ii) deur die flotasie- en sedimentasie metode waar aalwurmbesmette saad in agt verskillende NaCl konsentrasies geweek is, naamlik 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5% en 20%. Die effek van verskillende aalwurmdoders is ook onder semibeheerde omgewingstoestande in mikroplotte geëvalueer. Saad- en gal-aalwurms uit 12 van die 13 lokaliteite is geidentifiseer as *Subanguina wevelli*. Die bevolkingsdigtheid en PV het beide gewissel tussen 0.1 en 205 vir 'n 1g saadmonster, terwyl *S wevelli* op al die lokaliteite voorgekom het. Filogenetiese groepering van hierdie 12 *S. wevelli* bevolkings het "bootstrap" waardes van minder as 90% getoon. Hierdie 12 bevolkings het dus nie wesenlik van mekaar verskil nie en ook nie van die twee verwysingsbevolkings (wat uit die VSA verkry is) nie. 'n Lae gelykheids-waarde tussen die ITS areas van hierdie bevolkings asook die van die verwysingsbevolkings het hierdie indetifikasie verder ondersteun. Die *S.*

wevelli bevolking vanaf die verskillende lokaliteite word huidiglik in vivo op *E. curvula* gras (Ermelo variteit) vermeerder met die doel om volwasse aalwurms te bekom om molekulêre identifikasie van hierdie aalwurms verder te bevestig. Ten opsigte van die siftingsmetode het die getal *S. wevelli* in die saad wat op die verskillende grootte siwwe agergebly het, nie wesenlik van mekaar verskil nie. Saad op al drie hierdie siwwe het wel laer getalle aalwurms getoon as die ongesifte kontrole en het ook wesenlik verskil van dié ongesifte kontrole. Op die 250-µm sif het die skoonste saad (13% besmet) agtergebly, gevolg deur die op die 500-µm sif (28% besmet) en dan op die 600-µm sif (31% besmet). Die flotasieskeidingsmetode het getoon dat die verskillende NaCl konsentrasies wat gebruik is, nie wesenlik verskil het van die onbehandelde kontrole (skoon water) wat die getal *S. wevelli* betref nie. Die getal *S. wevelli* wat voorgekom het in die saad wat bly dryf het, het gewissel van 702 tot 1 952 vir die 2.5% en 20% NaCl behandelings, terwyl die getal aalwurms in die saad wat gesink het gewissel het tussen 84 en 392 vir die 12.5% en 5% behandelings. 'n Kombinasie tussen die siftings en 'n 15% NaCl konsentrasiemetode het steeds hoë getalle aalwurms in die saad opgelewer. Die saai van sogenaamde aalwurmbesmette saad sal dus steeds tot verdere besmetting in Eragrostis gras aanleiding gee. Die behandeling van *E. curvula* met saad wat bedek is deur 0.25mg avermectin per saad asook karbofuran (1.5g/m) en terbufos (0.66g/m) het die getalle van *S. wevelli* per 2g grass die meeste verlaag en het ook wesenlik van die onbehandelde kontrole verskil. Die aldikarb, kadusafos, etileendibromied, ethoprofos en oksamil behandelings het nog steeds relatiewe hoë aalwurmgetalle gehandhaaf en het verder nie wesenlik verskil van die onbehandelde kontrole nie. Die resultate wat tydens hierdie studie verkry is, wys daarop dat 'n enkele beheerstrategie nie genoeg is om te verseker dat die grassaad besendings vry van saad- en galaalwurms is nie. Om dié aalwurmprobleem suksesvol te beheer, is dit belangrik om na meer as een beheerstrategie, wat deel vorm van 'n geïntegreerde beheersisteem, in te sluit.

CHAPTER 1: General introduction

More than 40 nominal species of gall-forming plant-parasitic nematodes (PPN) that infect and parasitise both above- and belowground plant parts have been described (Subbotin *et al.*, 2004). Several anguinids (seed- and leaf-gall nematodes: SLGN) included in these 40 species are considered to be economically important in agricultural and horticultural crops and are also listed as quarantine pests in various countries (Subbotin *et al.*, 2004), including South Africa (Van den Berg, 1985). This study focused on determining the occurrence as well as the identification of SLGN parasitising *Eragrostis* spp. grass in South Africa. Identification included morphological as well as molecular methods such as the polymerase-chain reaction (PCR) and deoxyribonucleic acid (DNA) sequencing. In addition, management strategies were evaluated in this study in order to identify potential control strategies that could be used in integrated management systems.

1. *Eragrostis curvula* (Schrad.) Nees

1.1 Origin

The name "*Eragrostis*" originated from the Greek word "*Eros*" that means love and "*agrostis*" that refers to a type of grass (Van Oudtshoorn, 1999). *Eragrostis curvula* is commonly referred to as weeping lovegrass in South Africa and in the USA (Cook *et al.*, 2005). Other common names used for *E. curvula* are "boer love grass", "oulandsgras" and "african lovegrass" (Australia) (Bromilow, 2001; Cook *et al.*, 2005). Approximately 350 species of *Eragrostis* are recorded worldwide and most of them occur in subtropical areas (Skerman *et al.*, 2008). *Eragrostis* spp. constitute the biggest grass genus in southern Africa, containing 83 species, subspecies and varieties (Van Oudtshoorn, 1999). *Eragrostis curvula* occurs in southern Africa and northwards to east Africa. It was introduced throughout the tropics mainly as a fodder crop (Skerman *et al.*, 2008).

1.2 Classification

Weeping lovegrass is classified as follows (Accessed through GBIF Data Portal, www.gbif.net, 2008-05-27):

Kingdom: Plantae

Phylum: Angiospermae

Class: Monocotyledoneae

Order: Graminales

Family: Gramineae

Genus: *Eragrostis* von Wolf

Species: *E. curvula* (Schrad.) Nees

1.3 Anatomy

Eragrostis curvula is a tough, perennial grass that grows to a height of approximately 1200mm (Fig. 1.2) (Van Oudtshoorn, 1999). The leaf blades are ± 400mm long and ± 4mm wide, rolled or flat and concentrated mostly around the base of the plant (Van Oudtshoorn, 1999). Culms are unbranched and not easily compressed, with glabrous nodes and basal sheaths covered with long, dense hair (Skerman *et al.*, 2008). Spikelets are 4-10 mm long and 1-1.5mm wide, having a linear-oblong shape and are appressed to the branches (Skerman *et al.*, 2008). The inflorescence is ± 100-300mm long, branched and varies from open and spreading (throughout most of its distribution range) to contracted, with branches appressed to the main axis (Skerman *et al.*, 2008). *Eragrostis curvula* plants vary extremely in terms of morphology across a range of climatic conditions, which may lead to additional taxonomic divisions in future (Skerman *et al.*, 2008). Therefore, distinction between *E. curvula* and other species such as *E. chloromelos, E. lehmannian* and *E. rigidior* often proves to be complicated (Skerman *et al.*, 2008).

Figure 1.1. The (A) mature *Eragrostis curvula* plant, (B) inflorescence with spikelets, (C) seed with chaff and (D) seed without chaff.

1.4 Agronomy

Although *E. curvula* is mainly found in regions with high rainfall (500-1000mm) in the tropics and the subtropics (Skerman *et al.*, 2008), it is known to be drought tolerant (Cook *et al.*, 2005). The horizontal roots can spread to a distance of at least 1 metre and are, therefore, effective in competing for available soil water (Cook *et al.*, 2005). *Eragrostis curvula* establishes easily and may be grazed or cut earlier than other warm-season grasses during spring, making it a popular fodder crop (Mynhardt *et al.*, 1994).

The optimum temperature for growth of *E. curvula* is between 17°C and 32°C, although it can survive at 7°C (Cook *et al.*, 2005). *Eragrostis curvula* is a soil stabilizer that grows and produces well on a wide variety of soils (Torell *et al.*, 2000), but is best adapted to sandy and sandy loam soils (Dahl & Cotter, 1984). This also applies to South Africa where *E. curvula* grows on a variety of different soil types but is most abundant in sandy soils (Scheepers, 2005). *Eragrostis curvula* often grows in disturbed soil such as overgrazed or trampled grassland as well as on previously cultivated agricultural and horticultural fields (Van Oudtshoorn, 1999; Skerman *et al.*, 2008). *Eragrostis*

curvula, however, does not grow well in poorly drained soils and neither does it tolerate flooding (Cook *et al.*, 2005).

Eragrostis curvula is generally considered to be a relatively poor forage source for livestock during the summer season (Torell *et al.*, 2000) when it becomes unpalatable. Therefore, *E. curvula* is mainly harvested during spring (mid September to mid November in South Africa) in order to produce green forage (Drewes, 1985?). According to Sanderson *et al.* (1999) *E. curvula* (Ermelo variety) produces more forage dry matter than eight other warm-season perennial grasses in Texas (USA). However, it produces the lowest crude protein concentrations, with the nutritive value also being lower under improper management (Mynhardt *et al.*, 1994). This variety of *E. curvula* is also considered to produce good quality hay, especially when it is harvested before flowering, which guarantees high protein contents (Van Heerden, 2002).

A large proportion of *E. curvula* seed that was produced on the Highveld of South Africa during the 1980s was done by opportunistic farmers who established the crop with the primary objective of using it as an early-season grazing and hay source for livestock during winter (Rethman, 1988a). At present local *E. curvula* seed production is mainly done in the eastern parts of the Orange Free State (Bethlehem, Clarens, Harrysmith and Warden) as well as in the southern parts of Mpumalanga (Fig 1.2), with six varieties being commercially available namely, Agpal, Ermelo, PUK E3, PUK E 436, Umgeni, American leavy (Anonymous, 2008a). During the 2007/2008 seasons 192 375kg *E. curvula* seed at a value of R 12.76 million were produced in South Africa (Anonymous, 2008b).

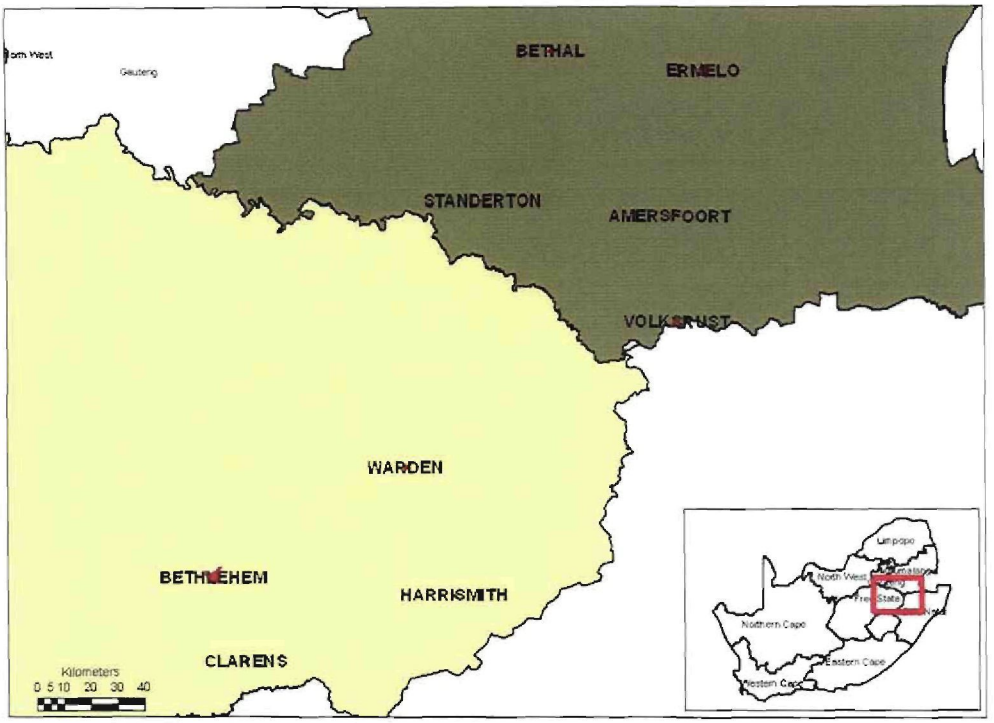

Figure 1.2. Main *Eragrostis curvula* production areas in the Free State and Mpumalanga Provinces of South Africa (compiled by Durand[1]).

Eragrostis curvula (Ermelo variety) has also been used worldwide in crop-sequence rotations to reduce populations of economically important PPN such as root-knot (*Meloidogyne* spp.) and root-lesion (*Pratylenchus* spp.) nematodes in tea, potato and tobacco plantings successfully (Fourie *et al.*, 1998; Cook *et al.*, 2005). In South Africa this variety is a poor host of *M. chitwoodi* and could be used with success by potato farmers to optimise tuber quality and income (Fourie *et al.*, 1998). However, in fields where SLGN are present, *Eragrostis curvula* lovegrass should not be used for this purpose.

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2. Plant-parasitic nematodes

Nematodes are multicellular, unsegmented worm-like organisms and could be found virtually anywhere on earth, including aquatic as well as terrestrial environments (Decraemer & Hunt, 2006). Despite the diverse ecological niches nematodes occupy they are generally similar in structure (Luc *et al.*, 1990). These organisms can be either free-living in marine, freshwater or soil environments or could parasitise plants, humans and animals (Maggenti, 1981).

All PPN belong to the phylum Nematoda, with most of the important parasitic genera belonging to the order Tylenchida (Decraemer & Hunt, 2006). According to these authors nematodes are currently divided into two major classes, namely i) the Chromadorea and ii) Enoplia. The class Chromadorea includes free-living as well as PPN (Siddiqi, 2000) under the order Rhabditida, the latter previously having been known as the order Tylenchida (Luc *et al.*, 1990, Kleynhans *et al.*, 1996). The class Enoplia comprises only PPN grouped under the order Dorylamida (Decraemer & Hunt, 2006).

Annual worldwide losses caused by nematodes in life-sustaining crops that include grains, legumes, vegetables, tropical and subtropical crops are estimated at approximately 10 percent (Whitehead, 2002). In monetary terms Chen *et al.* (2004) estimated annual worldwide crop losses attributed to PPN to be approximately $78 billion. Knowledge of the relationship between crop yield, quality and population levels of PPN is, therefore, fundamental to the application of informed and rational nematode management decisions (Ferris, 1984; Schomaker & Been, 2006). For this reason it is also of utmost importance that the identification of PPN species is accurate (Zijlstra *et al.*, 2000; Subbotin & Moens, 2006).

2.1 General morphology and biology

Nematodes are generally vermiform except in some genera, usually ranging from 0.2 to 1mm in length (Luc *et al.*, 1990) and 15 to 35µm in width (Argios, 1997). However, some PPN could be more than 3mm long (Luc *et al.*, 1990). In some PPN species females lose the vermiform shape and become obese or even globose (Luc *et al.*, 1990), while males remain vermiform. This

phenomenon is known as sexual dimorphism and is present in genera such as *Meloidogyne* (root-knot), *Heterodera* and *Globodera* (cyst), *Rotylenchus*, *Cactodera* and *Meloidodera* (Luc *et al.*, 1990). Despite their diversity in lifestyle and feeding habit, PPN have a relatively conserved body structure (Decraemer & Hunt, 2006). A typical nematode body consists of an external wall and internal cylindrical digestive system. The body wall (cuticle) and digestive system are separated by a pseudocoelomic cavity that is filled with fluid, which is under pressure (Decraemer & Hunt, 2006).

The nematode body is more or less transparent and is covered by a colourless cuticle that is produced by the hypodermis (Agrios, 1997; Decraemer & Hunt, 2006). The hypodermis consists of living cells and extends into the body cavity as four chords that separate four bands of longitudinal muscles and enable nematodes to move (Agrios, 1997, Decraemer & Hunt, 2006). Specialised muscles that are used during feeding are also found in the mouth region along the digestive tract and the reproductive structures (Agrios, 1997; Decraemer & Hunt, 2006). The pseudocoelom of nematodes acts as a hydrostatic skeleton in which the digestive, reproductive and excretory systems are nested (Luc *et al.*, 1990; Decraemer & Hunt, 2006). All PPN have either a hollow or solid stylet or spear, which they use to penetrate plant cells and withdraw nutrients from the cells (Agrios, 1997; Decraemer & Hunt, 2006).

The reproductive system of nematodes is well developed, with one or two ovaries and a uterus that terminates in a vulva in females (Agrios, 1997; Decraemer & Hunt, 2006). Male nematodes have testes, seminal vesicles, copulatory spicules and in some species also a bursa (Agrios, 1997; Decraemer & Hunt, 2006).

2.2 Life cycle

Nematodes typically have four juvenile stages (J1, J2, J3 and J4) between egg and adult, with intervening moults allowing increase in size, both in width and in length (Luc *et al.*, 1990). Eggs may be deposited singly or in masses (Decraemer & Hunt, 2006). As soon as conditions are optimal, juveniles will hatch from the eggs (Agrios, 1997). Juveniles grow in size and each

particular juvenile stage is ended by a moult (Agrios, 1997). Females could produce fertile eggs either after mating with a male (sexual) or in the absence of males (parthenogenetically) (Agrios, 1997). The life cycle of SLGN in general is completed in approximately 113 days (Luc *et al.*, 1990), but it varies considerably for different genera and species. The J2 represents the infective stage of most PPN since it is optimally adapted for dispersal as well as for surviving adverse conditions (Decraemer & Hunt, 2006).

2.3 Behaviour and feeding of PPN

In terms of habitat and feeding patterns, PPN are either ectoparasitic or endoparasitic (Decraemer & Hunt, 2006). Ectoparasitic nematodes do not normally enter root tissue but feed only from the outside on the cells near the root surface (Agrios, 1997). Endoparasites, on the other hand enter a plant and then migrate within the host or become sedentary and feed from within the host (Agrios, 1997). Both endo- and ectoparasites can be migratory, which imply that they live freely in the soil and feed on plants without becoming attached or sedentary (Agrios, 1997). PPN infect below-ground as well as above-ground parts of plants.

2.4 Symptoms

The most general, universal symptom caused by PPN in host plants is a reduction in plant growth and ultimately in yield compared with that of healthy, uninfected plants (Manzanilla-López *et al.*, 2004; Luc *et al.*, 2005). Symptoms, above- and below-ground may vary according to the parasitic habits of particular PPN and their host-parasite relationships, as well as other factors such as age and physiological condition of the host plants (Manzanilla-López *et al.*, 2004). Symptoms caused by PPN nematodes on the above- and below-ground parts of plants are often subtle and difficult to distinguish from damage by other pests, diseases or other factors such as fertilizer deficiency, drought, excessive rainfall, etc. (Manzanilla-López *et al.*, 2004; Luc *et al.*, 2005). Above-ground symptoms expressed by host plants due to infection by PPN often include stunting, yellowing, wilting, early senescence and poor crop growth, yield or quality or galls on seeds/leaves/stems caused by SLGN (Manzanilla-López *et al.*, 2004; Luc *et al.*, 2005). Below-ground symptoms

vary considerably and may include knots/galls on roots/tubers/pods caused by root-knot nematodes, necrotic lesions on roots caused by lesion nematodes or on pods, e.g. by the groundnut pod nematodes (Manzanilla-López *et al.*, 2004; Luc *et al.*, 2005). It is, therefore, important to identify symptoms caused by PPN accurately in order to address and manage the nematode problem successfully.

3. Seed- and leaf-gall nematodes (*Anguina*/*Subanguina* spp.)

Since this study focuses on the identification, occurrence and management of SLGN, their importance as parasites of *E. curvula* grass will be addressed by discussing their (i) position in the nematode kingdom (classification), (ii) morphology, biology and life cycle, (iii) economic importance, (iv) identification and (v) control.

3.1 Classification

The Anguinidae family represents mycophagous PPN that parasitise both below- and above-ground plant parts (Kleynhans *et al.*, 1996; Duncan & Moens, 2006). This family comprises three genera, namely *Anguina*, *Subanguina* and *Ditylenchus* (Kleynhans *et al.*, 1996; Duncan & Moens, 2006). This study, however, only focuses on SLGN, which include *Anguina* and *Subanguina* spp. The most recent classification for *Anguina* spp. Scopoli, 1777 was published by Siddiqi (2000) as reported by Decreamer & Hunt (2006) and is as follows:

Phylum: Nematoda
Class: Chromadorea
Order: Rhabditida
Suborder: Tylenchina
Superfamily: Tylenchoidea
Family: Anguinidae
Subfamily: Anguininae
Genus: *Anguina/Subanguina* spp. Scopoli, 1777
(wheat or seed- and leaf-gall nematodes)

3.2 Identification

The economically most important *Anguina* species is *A. tritici*, which was the first PPN ever reported during 1743 from wheat seed galls (Fig. 1.3) in the UK (Duncan & Moens, 2006). All *Anguina* species identified to date inhabit seeds, leaves and/or other above-ground parts of grain crops, as well as forage grasses and cause gall formation on these plant parts (Agrios, 1997). Eight of these anguinid species are important in terms of regulatory aspects (Duncan & Moens, 2006).

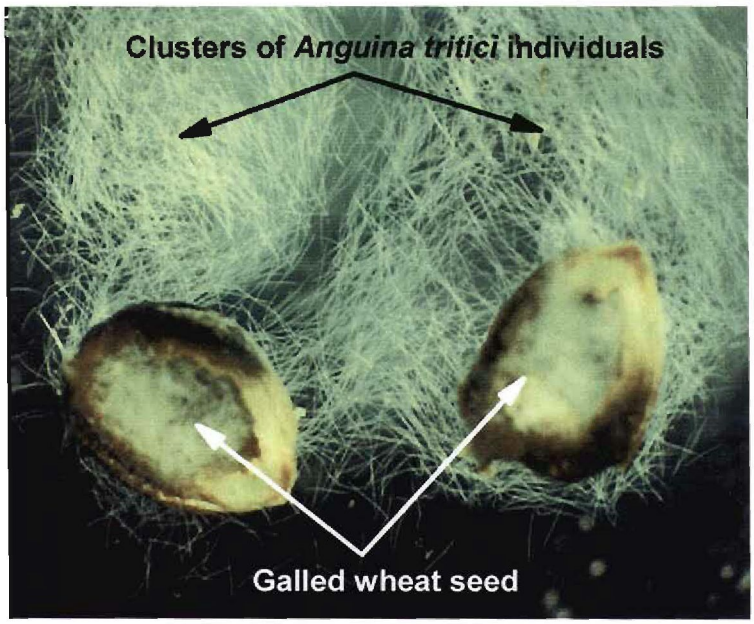

Figure 1.3. *Anguina tritici* second-stage juveniles (J2) emerging from galled wheat seed (Ferris, 1999).

According to Powers *et al.*, (2001) 11 valid species of *Anguina* have been identified following the latest classification system of Siddiqi (2000). However, it has not been possible to describe species of the genus *Anguina* on morphological grounds only (Riley *et al.*, 1988). The reason is that the number of species varies considerably between classification systems and corresponds with the recognition or not of various genera or subgenera (Powers *et al.*, 2001). For example, *S. wevelli* (Van den Berg, 1985) Ebsari originally described from weeping lovegrass (*Eragrostis curvula*) in South Africa by morphological identification alone was synonymised with *Anguina agrostis* (Steinbuch, 1799) Filipjev, 1936 (Chizhov & Subbotin, 1990). The latter was known as the bentgrass nematode (Chizhov & Subbotin, 1990).

However, Ebsari (1991) did not acknowledge this synonymy and placed the species in the genus *Subanguina* Paramonov, 1967. Siddiqi (2000) reinstated the latter species in the genus *Anguina*. These taxonomic discrepancies cause controversy when regulatory issues arise (Powers *et al.*, 2001; see paragraph 3.8.1).

Another underlying cause of taxonomic confusion is nondescriptive morphology that complicates anguinid identification (Powers *et al.*, 2001). Often only J2 and J3 are present inside seed galls, which further complicates identification of anguinids by means of traditional morphological techniques (Powers *et al.*, 2001). Juveniles lack certain key diagnostic features such as gonads, stylets, etc. (Heyns, 1971) and this way complicates proper and accurate morphological identification.

DNA-based diagnostics developed during the past decade provided an attractive solution in addressing problems associated with morphological identification of PPN (Subbotin *et al.*, 2000), particularly with regard to SLGN. Not only are qualitative differences between PPN important for diagnosis (i.e. genus, species or race present in a sample) but nematodes must also be quantified in order to make correct preventative management decisions (Roberts, 1994; Subbotin & Moens, 2006). Powers *et al.* (2001) furthermore confirmed through ITS1 nucleotide sequencing that *S. wevelli*, *A. funesta* and *A. agrostis* that were regarded as synonyms, are all separate species. The fact that shipments of *E. curvula* seed infected with *S. wevelli* imported from South Africa have been intercepted in the United States on several occasions (personal communication, Mr. Tony Siebert[2]) emphasises the importance of implementing molecular identification for confirmation of morphological identification of anguinids.

[2] Mr. Tony Siebert, Advance Seed, 26 College Laan, Potchefstroom, 2531, South Africa.

3.3 Morphology and biology

Anguina spp. specimens are relatively large compared to other PPN, with several species being approximately 5mm (Hunt *et al.*, 2005). Females are generally rather stout, with '*a*'-values[3] usually close to 20. Males have more slender bodies that are swollen near the middle due to extensive development of the gonads (Jenkins & Taylor, 1967). When specimens are investigated under a low magnification dissection microscope males are observed as being only slightly curved, while females usually are coiled ventrally (Jenkins & Taylor, 1967; Hunt *et al.*, 2005). The cuticle of *Anguina* spp. is finely striated and lateral fields are either plain or marked with, four or more incisures (Jenkins & Taylor, 1967). The lip region is narrow, annulated, slightly flattened and offset from the body contour (Jenkins & Taylor, 1967). The stylet of most *Anguina* spp. is short, approximately 8µm to 10µm in length, with well-developed, rounded basal knobs (Jenkins & Taylor, 1967). Although the oesophagus is typically tylenchoid the basal bulb may be swollen, irregularly shaped and the dorsal gland may overlap the anterior part of the intestine (Jenkins & Taylor, 1967; Geraert, 2006). The structure of the gonads is of prime diagnostic value since oocytes and spermatocytes are produced in multiple rows in the formation of a rachis (Jenkins & Taylor, 1967; Kleynhans *et al.*, 1996). In females the ovary is single, typically anteriorly reflexed once or twice and has a typical rudimentary, post-vulval uterine branch (Jenkins & Taylor, 1967; Geraert, 2006). The spermatheca is an elongated sac consisting of 16 cells containing large sperm cells (Geraert, 2006). In males the testis is well developed with one or more flexures (Hunt *et al.*, 2005). Spicules are short, thick and paired but not fused and arcuate, with rather wide blades (Jenkins & Taylor, 1967; Geraert, 2006). The gubernaculums are plain and trough-like, with the bursa located anterior to the spicules, stretching nearly to the tail tip (Jenkins & Taylor, 1967). The tail is more or less elongated to filiform (Geraert, 2006).

3.4 Life cycle

Similar to the majority of other PPN species SLGN also has four juvenile stages (Willmott *et al.*, 1972). *Anguina* and *Subanguina* spp. generally

[3] 'a'-value – total body length divided by maximum body width (Wilmott *et al.*, 1973).

produce one generation per year and are dispersed by movement of infected seed or other above-ground plant material through wind, implements and others (Jenkins & Taylor, 1967). J1 moult after hatching from eggs and develop into J2, which infect the host plant (Willmott *et al.*, 1972). SLGN spp. generally overwinter in seed galls as J2 (Agrios, 1997). Infected galls fall to the ground and when environmental conditions are optimal the galls soften and infective J2 are released (Agrios, 1997). J2 use the water film on the surface of a host plant to move upwards towards the tightly compacted leaves near the growing point (Agrios, 1997). Here it will feed ectoparasitically until the inflorescence begins to develop (Agrios, 1997). J2 of *A. tritici* infect the growing point of wheat after they have moved out of moistened seed galls (Duncan & Moens, 2006). The stem elongates and as it grows it carries J2 upward along with the developing ear (Duncan & Moens, 2005). The J2 then enter a floral primordial soon after the onset of development of the inflorescence and develop into J3, J4 and subsequently into adult females or males (Fig. 1.4) (Agrios, 1997). Penetration and feeding of juveniles on primordial tissue result in the formation of galled seed (Maggenti, 1981). Each infected floral primordium becomes a galled seed and may contain 80 SLGN (Agrios, 1997). Inside the newly formed gall the females deposit their eggs over a period of several weeks and die soon thereafter (Agrios, 1997). After hatching J1 emerge and moult to develop into a J2. These J2 are resistant to desiccation and can survive in a gall for periods up to 30 years (Agrios, 1997). The life cycle of *A. tritici* is completed within approximately 113 days, with the adult stages being reached between 68 and 102 days after grass has germinated (Luc *et al.*, 1990).

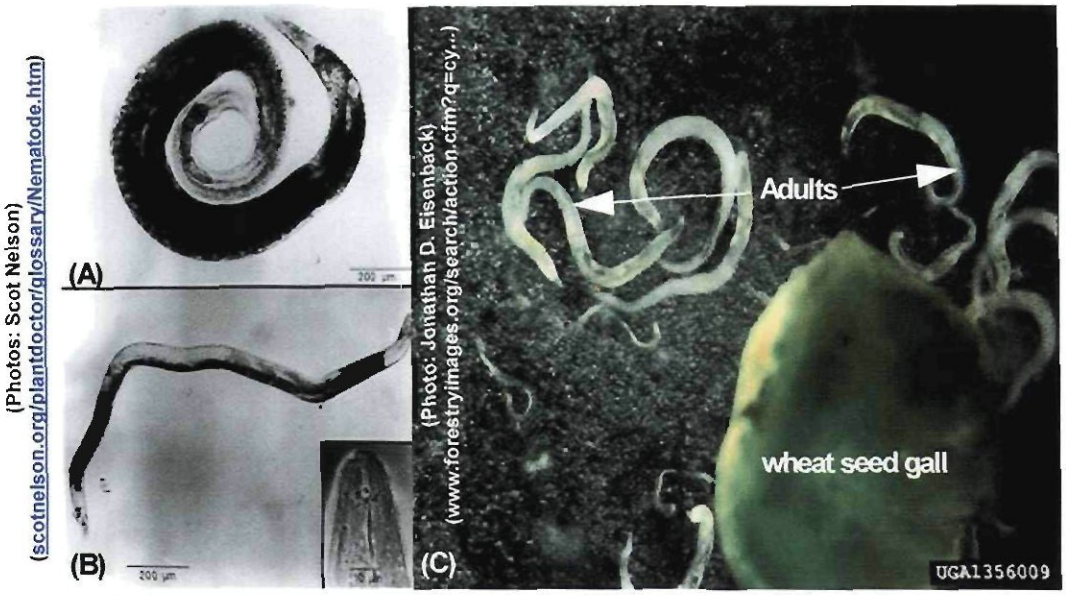

Figure 1.4. An *Anguina* spp. (A) female, (B) male and (C) adults emerging from infected wheat seed kernel.

3.5 Symptoms of SLGN

The most noticeable symptom of infection by *A. tritici* is the presence of thickened, dark galls (Fig. 1.5A) instead of sound seed kernels (Ferris, 1999). Wheat plants infected with *A. tritici* also mature more slowly compared to uninfected ones and produce smaller ear heads (Fig. 1.5B & 1.5C) (Ferris, 1999). The ectoparasitic feeding habit of *A. tritici* also usually causes emerging leaves of wheat plants to be rolled, curled or spiralled (Ferris, 1999). However, the leaves could straighten out after 30-45 days after SLGN infection and then appear normal, except for faint ridges that could be observed on the surface (Fig. 1.5D) (Mc Donald & Nicol, 2005).

Figure 1.5. *Anguina tritici* infected, (A) dark wheat galls and (B & C) wheat ear heads with (D) twisted and crinkled leaves as a result of the infection by this nematode.

No obvious symptoms can be detected when *A. agrostis* attacks bentgrass although the effect on the inflorescence (Fig. 1.6A) is a reduction in seed yield (Courtney & Howell, 1952; Willmott *et al.*, 1973). Seed galls caused by SLGN are more difficult to detect in grasses as these galls are covered by lemmas and paleas (Ferris, 1999), which for example is the case with *A. agrostis* (Fig. 1.6B) and *A. funesta* (Fig. 1.6C). Infection of grass by *S. wevelli* could be detected due to the presence of characteristic dark seed galls (Fig. 1.6D) that are harvested together with the healthy uninfected seed of *Eragrostis curvula* (Fig. 1.6C) (Inserra *et al.*, 2003).

Figure 1.6. Various symptoms associated with SLGN infection, where (A) the deformed inflorescence of *Agrostis* spp. as well as (B) the galled spikelets are visible when infected with *Anguina agrostis*; (C) annual rye grass seed (*Lolium rigidum*) (i) with chaff and (ii) without chaff as well as (iii) a seed gall infected with *Anguina funesta*; (D) dark galled seed of *Eragrostis curvula* infected with *Subanguina wevelli*.

3.6 Survival

A wide range of PPN is able to survive unfavourable conditions by suspending their development and remaining in a dormant state until conditions become favourable again (Wright & Perry, 2006). Anhydrobiosis is such a survival strategy and is characterised by adaptations by these parasites to enable structural and biochemical changes (Wright & Perry, 2006). The latter changes are prerequisites for the survival of such PPN during prolonged periods of unfavourable conditions (Wright & Perry, 2006). Coiling and clumping (or aggregation) occurs when the body fluid of nematodes that contains water is replaced by glycerol and trehalose (Barrett, 1991; Wright & Perry, 2006). Osmotic stress and increase in temperature also reduce the surface area of the bodies of nematodes exposed to drying conditions (Wright

& Perry, 2006). Anhydrobiotic nematodes rehydrate when placed in water but may take some time to resume their normal activities (Barrett, 1991). This rehydration period normally takes a few hours, but can vary from less than an hour to several days, increasing with the intensity of the anhydrobiotic state experienced by SLGN (Barrett, 1991). Permeability of the cuticle is also reduced in anhydrobiotic PPN (Barrett, 1991).

Some of the most successful anhydrobiotic organisms are SLGN, particularly *A. tritici* (Womersley *et al.,* 1982). Chances of SLGN survival are best when the desiccation process is slow since most species are killed when it happens too quickly (Barrett, 1991).

3.7 Economic importance

Except for *A. tritici* other SLGN such as *A. agrostis*, *A. funesta* and others (Table 1.2) are considered to be of the economically most important agricultural and quarantine pests in several countries (Subbotin *et al.*, 2004). *Anguina tritici* is of economic importance in Brazil (Maggenti, 1981), Eastern Europe, India, West Asia (Swarup & Sosa-Moss, 1993), Northern Iraq (Al-Talib *et al.*, 1986), the Pacific North West of the USA (Griffin, 1984) and the Middle East (Maggenti, 1981) where it mainly infects wheat. Since 2000 *A. tritici* has been recognised as a quarantine pest and is regulated in 24 countries (Hockland *et al.*, 2006). *Anguina tritici* can cause wheat yield losses of up to 65% (Maggenti, 1981), which is in agreement with a yield loss figure of 60% that could result when infected seed are planted (Duncan & Moens, 2006). There was also a report of *A. agrostis* that reduced yields of *Astoria* spp. bentgrass by 50 – 75% in Oregon, USA (Jensen, 1961).

The SLGN problem is aggravated due to is association with both fungal and bacterial diseases in graminaceous hosts (Riley & McKay, 1990; Mc Donald & Nicol, 2005). *Anguina funesta* in association with a coryneform bacterium (*Clavibacter* spp.) causes a disease known as annual ryegrass toxicity (Riley *et al.*, 1988) resulting in the death of livestock. It was first recorded in 1956 in South Australia and then during 1980 in South Africa (McKay, 1993). At present *A. agrostis* together with the bacterium *Clavibacter toxicus* are

responsible for annual ryegrass toxicity and the death of livestock in South Africa, especially in the Western Cape where ryegrass is used for pasture (personal communication, Dr. Kitching[4]).

Table 1.1: Different seed- and leaf-gall nematodes (SLGN) associated worldwide with the family Gramineae (Subbotin *et al.*, 2004).

SLGN species	**Host-plant**	**Part of host plant where galls are formed**	**Country**
A. agropyri Kirjanova (1955)	*Elymus repens*	Basal stem	Estonia
A. agrostis (Steinbuch, 1799)	*Agrostis capillaries* *Lolium perenne*	Seed	New Zealand, USA, Belgium, Russia, South Africa
A. australis Steiner (1940)	*Ehrharta longiflora*	Leaf	Western Australia
A. funesta (Price, Fisher and Kerr, 1979)	*Lolium rigidum*	Seed	South Australia, Western Australia
A. graminis (Hardy, 1850)	*Festuca rubra*	Leaf	Russia
A. tritici (Steinbuch, 1799)	*Triticumm aestivum*	Seed	Western Australia
A. askenasyi (Bütschli, 1873)	*Calliergon cuspidata*	Terminal	Estonia
S. wevelli	*Eragrostis curvula*	Seed	South Africa
A. woodi	*Ehrharta villosa*	Stem	South Africa

Present management approaches for reducing PPN are more holistic and constitute a broad combination of tools, which is based on farmers' needs rather than scientific ideologies (Sikora *et al.*, 2005). The ultimate goal in terms of PPN control is a reduction in population levels of these parasites together with an increase in crop yields and/or quality at levels that are cost-effective for producers (Sikora *et al.*, 2005).

[4] Dr. Kitching, Western Cape Provincial Veterinary Laboratory, Private Bag X 5020, Stellenbosch 7599, Republic of South Africa.

Previously SLGN belonging to the genus *Anguina* have been reported to infect different grass spp. in southern Africa, such as *Anguina* spp. infecting *Eragrostis tef* in Zimbabwe, *Hyparrhenia* spp. in Zambia and Malawi as well as *Pennisetum typhoides* in Zimbabwe and Malawi. *Anguina tritici* was also reported to parasitise *Triticum aestivum*, while *A. tumefaciens* were recorded to infect *Cynodon transvaalensis* as well as lawns in South Africa (Keetch & Buckley, 1984). In the Western Cape Province of South Africa *A. agrostis* was found in seed of *Lolium* spp. (personal communication, Professor A.J. Meyer[5]). *Subanguina wevelli* was locally first identified by Van den Berg (1985) from seed samples of *E. curvula* collected from two localities near Harrysmith and Reitz in the Free State Province and at one locality Bethal in Mpumalanga Province. Dr. Swart[6] also recorded the presence of *S. wevelli* during 2000 in seed from *E. curvula* grown in the Mpumalanga and the Free State Provinces (personal communication). The survey that was conducted during this study showed that at present *S. wevelli* is still occurring in the Bethal area, but has also been found at localities such as Amersfoort and Standerton (Mpumalanga Province) as well as Potchefstroom (North West Province).

3.8 Control of SLGN

3.8.1 Quarantine

The importing of soil, growing media and packaging material may harbour PPN that are listed as unwanted organisms in international trade due to their potential threat to certain crops (Hockland *et al.*, 2006). The SLGN *A. tritici*, for example is a quarantine organism (Hockland *et al.*, 2006). Quarantine measures against SLGN are, however, complicated by morphological identification of various species. These include *S. wevelli*, *A. agrostis* and *A. funesta*, which are considered to be identical. The synonymy of *S. wevelli* and *A. agrostis* also caused a quarantine status to be instated on SLGN found in *E. curvula* since *A. agrostis* and *A. funesta* were previously regarded synonyms (Siddiqi, 1985). The possibility of this nematode being identical to

[5] Professor A.J. Meyer, P.O. Box 431, Kuilsrivier, 7579, South Africa.

[6] Dr. Swart, Biosystematics Division, ARC-PPRI, Private Bag X134, Queenswood, 0121, South Africa.

A. funesta as claimed by Krall (1991) restricts the import/export of infected seed on a global basis (Powers *et al.*, 2001). The use of molecular techniques, however, these days addresses the latter problem (see paragraph 3.2.2).

3.8.2 Mechanical separation techniques

The major strategy generally proposed for preventing infection of host plants with SLGN is use of uninfected seed (Whitehead, 2002). Mechanical sieving and flotation of contaminated SLGN-infected seeds in brine (diluted NaCl solution) or hot-water treatment (Brown & Kerry, 1987) have been used successfully to eliminate *A. tritici* galls from wheat in Europe and other countries. According to Paruthi and Bhatti (1992) 78% to 92% of *A. tritici* galls were removed by sieving, using a 3-mm-mesh sieve. Although mechanical sieving of seed also removed the majority of the *A. agrostis* galls from seed of *Agrostis* spp., control of these nematodes is more effective when sieving is combined with hot water treatment (Ferris, 1999).

3.8.3 Crop rotation

Crop rotation is one of the oldest and most widely used methods to reduce pests and diseases in agricultural and horticultural crops (Hooper & Evans, 1993). One-year crop rotations with non-host plants were reported to be successful in removing *A. tritici* and *A. agrostis* individuals from infested soil in the USA, Europe, Australia, India and Ethiopia (Ferris, 1999).

3.8.4 Chemical control

In terms of nematicide use to control anguinids, Willmott (1973) reported an effective reduction in *A. tenius* numbers in grass seed (± 12% moisture content) with methylbromide fumigation of seed assignments. No pronounced adverse effects were observed in terms of the germination of these fumigated seed (Willmott, 1973). Spraying of galled wheat seed with ethyl parathion also eliminated SLGN galls from seed assignments (Whitehead, 2002). Since the latter treatment entails an extreme health risk to humans, it is not used or recommended (Whitehead, 2002). The use of boinematicides such as neem kernel powder also resulted in a substantial reduction in *A. tritici* numbers

(Gokte & Swarup, 1989). A similar reduction in *A. tritici* numbers was observed when infected soil was treated with leaf extracts *Catharanthus roseus* (Madagascar periwinkle) (Whitehead, 2002). Herbicide application also proved effective in reducing *A. agrostis* populations in grass since it suppresses flowering formation (Courtney *et al.*, 1962; Willmott, 1973).

3.8.5 Timing of planting or harvesting

Since PPN-population densities fluctuate during every season, it is possible to reduce their damage by manipulating planting dates to avoid periods of peak PPN activity during planting (Hooper & Evans, 1993; Viaene *et al.*, 2006). Because certain PPN such as SLGN only have one reproductive cycle per cropping season they can be controlled by deliberately harvesting the crop before the nematode's life cycle has been completed (Hooper & Evans, 1993). Venter *et al.* (1992) demonstrated the efficacy of differential planting dates in suppressing the SLGN *Ditylenchus africanus* numbers in groundnut pods and seeds.

Although not always directly applicable to SLGN, other commonly used strategies to minimise the effect of PPN in agricultural and horticultural crops will be discussed below. These strategies could possibly contribute towards SLGN control when included as part of a management programme.

3.8.6 Physical control strategies

Of all the non-chemical strategies available to manage PPN physical measures are probably being used most frequently, particularly by resource-poor farmers (Bridge, 1996). These most-used methods include heat, flooding, soil tillage, clean fallow and organic amendments (Bridge, 1996; Whitehead, 2002; Sikora *et al.*, 2005; Viaene *et al.*, 2006).

Heat

Effective control of PPN has been achieved by heating of the soil, infected plants or plant material to the thermal death-point of the PPN (Viaene *et al.*, 2006). Most PPN are killed when exposed to temperatures of 53°C to 55°C for 20 minutes (Viaene *et al.*, 2006). Methods used to effectively kill

nematodes by heat include dry heat, steam, hot-water drenches and hot-water dips (Whitehead, 2002; Sikora *et al.*, 2005; Viaene *et al.*, 2006). High ternperature can, however, also adversely affect germination of seed, tubers or bulbs (Halbrendt & LaMondia, 2004).

Flooding

PPN densities can be reduced significantly when soils are flooded for prolonged periods (Sikora *et al.*, 2005). The duration of the flooding to be effective in killing various species of PPN needs to be determined before it could be applied as an effective means for nematode control (Sikora *et al.*, 2005). Ill-considered flooding could have undesired effects on crops since it could drastically reduce soil oxygen concentration, pH and could cause an increase in the leaching or spreading of toxic substances (Viaene *et al.*, 2006).

Soil tillage

Soil tillage generally reduces the densities both of target PPN as well as those of secondary pests, while it also eliminates alternative weed hosts and volunteer plants from a previous crop (Sikora *et al.*, 2005). Where the practice is economically justifiable, repeated tilling of soil at regular intervals of 30 days during hot and dry seasons between crops can significantly reduce PPN densities in the upper 30-cm soil layer due to desiccation of eggs and juveniles (Sikora *et al.*, 2005). Soil water loss under these conditions, however, poses a serious risk to the crop in question. Therefore, the use of tillage as a nematode control measure should always be considered against all conflicting interests of maintaining a reduced-tillage production system.

Fallow

Fallows, also referred to as uncropped lands could be applied in various forms namely bush, clean (bare) and grass fallows (Bridge, 1996). Fallow is usually referred to when land is unused for a season or even longer after ploughing and harrowing and is kept free of volunteer crop plants and weeds, thus reducing PPN numbers (Sikora *et al.*, 2005). Although a clean fallow is best for reducing nematode populations its use implies no crop production for a

large part of a growing season (Keetch & Heyns, 1982; Bridge, 1996). Furthermore, such a field will be exposed to rain and wind erosion as well as leaching of essential nutrients (Keetch & Heyns, 1982; Sikora *et al.*, 2005). Grass fallows may reduce PPN populations, particularly root-knot nematodes, but could assist in maintaining certain PPN, for example those hosted by certain weeds and also SLGN.

Organic amendments

Organic amendments entail that organic material such as plant residues, industrial or animal waste products is incorporated into the soil (Sikora *et al.*, 2005). The addition of organic material to soil has generally proved to cause a decrease in population densities of PPN (Keetch & Heyns, 1982; Sikora *et al.*, 2005), while improving growth and yield of nematode-infected crops. Addition of organic material further improves (i) soil structure, (ii) nutrient supply and (iii) provides substrates for the multiplication of beneficial organisms such as PPN antagonists (fungi, bacteria, and omnivorous nematodes) in the soil (Ferras & De Freitas, 2004). Thus, stronger plants with improved tolerance to PPN are grown (Hooper & Evans, 1993).

3.8.7 Host-plant resistance

Host-plant resistance is another strategy for suppressing PPN numbers successfully and is also cost-effective and environmentally friendly (Starr *et al.*, 2002; Cook & Starr, 2006). Plant resistance is defined as the ability of a host-plant to inhibit nematode development and reproduction relative to that of a susceptible variety (Sikora *et al.*, 2005) and refers to the efficacy of the plant to maintain a given PPN population (Cook & Starr, 2006). Although host-plant resistance is currently only available for several PPN species and for a limited number of crops, a great need exists for development of resistance to more PPN species in a wider range of crops (Cook & Starr, 2006).

3.8.8 Biological control

Biological control could be defined as the management of PPN by means of living organisms such as predatory nematodes or microbes (fungi/bacteria) that adversely affect the establishment, development or survival of a PPN population (Viaene *et al.*, 2006). Establishment of these organisms in fields

under harsh environmental conditions has, however, not yet been developed to such and extent that it is always a viable option for control of PPN (Chen & Dickson, 2004).

3.9 The SLGN problem in South Africa

The direct economic risk posed by *S. wevelli* is, rated low in the USA (Inserra *et al.*, 2003) probably due to limited distribution of this parasite. The presence of *S. wevelli* in seed of *Eragrostis* spp. in South Africa is, however, currently of great economic importance to the local grass seed industry and producers. A total of 800 tonnes of seed with an estimated value of R 11 million had been produced in South Africa both for the export and local markets since 2001. These production figures could, however, not be met due to the profound SLGN problem experienced by local producers (personal communication, Mr. Tony Siebert[2]). For this reason the present study, which was requested by the local seed industry, became a priority in terms of addressing and finding practical solutions to this problem.

5. Objectives

The objectives of this study were to i) conduct a survey in the *Eragrostis* spp. production areas of South Africa to assess and quantify the extent of the SLGN problem (prominence, population densities and frequency of occurrence), ii) determine which SLGN species are involved by means of morphological and molecular identification, iii) investigate mechanical, commercialisable techniques to separate galled seed from uninfected seed and iv) evaluate various nematicides for their efficacy in reducing SLGN in a microplot trial.

The outcome of this study may assist in limiting the spread of SLGN in South Africa as well as in providing measures to producers and the seed industry for safe trading in uninfected grass seed on an international basis.

CHAPTER 2: Occurrence, distribution and molecular identification of seed- and leaf-gall nematodes (SLGN) in *Eragrostis curvula* seed

1. Introduction

The seed- and leaf-gall nematode (SLGN) *Subanguina wevelli* was found and described in South Africa in 1985 from seed of weeping lovegrass (*Eragrostis curvula*) (Kleynhans *et al.*, 1996) produced in the areas of Harrysmith and Reitz in the Free State Province. This species was also found in seed from other *E. curvula* crops that were produced in the eastern Mpumalanga and eastern part of the Free State (Ermelo, Amersfoort and Warden) during 2000. It became an economically important parasite, limiting seed export (personal communication, Mr. Tony Sibert, Advance Seed[1] and Dr. A. Swart, ARC-PPRI[2]).

In the past identification of PPN relied only on morphology, which had been the principal method used for species description (Riley *et al.*, 1988). However, in recent years molecular techniques proved to be more useful for resolving problems experienced in distinguishing between PPN, particularly in the case of *Anguina* spp. (Riley *et al.*, 1988). In terms of clarifying the classification of the Anguinidae, the use of deoxyribonucleic acid (DNA) techniques was demonstrated by Subbotin *et al.* (2004) and Powers *et al.* (2001). These authors, for example emphasized the use of internal transcribed (ITS) sequences for phylogenetic analyses of the Anguinidae.

Although preventative rather than corrective control strategies are recommended in PPN management, most fields currently under *E. curvula* production already maintain unacceptably high levels of SLGN infections. Re-infestation of these fields or infestation of SLGN-free fields generally occurs when *E. curvula* seed infected with these nematodes is planted. Hence the situation is aggravated to such an extent that the local Eragrostis seed market

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[1] Mr. Tony Siebert, Advance Seed, 26 Collage Laan, Pothcefstroom, 2531, South Africa.

[2] Dr. Antoinette Swart, Biosystematics Division, ARC-PPRI, Private Bag X134, Queenswood, 0121, South Africa.

is currently seriously hampered in terms of sustainable production, while export of seed is also adversely affected (personal communication, Mr. Tony Siebert, Advance Seed[1]).

2. Specific objectives

The objectives of this study were to i) conduct a survey in the *Eragrostis* spp.-production areas of South Africa in order to assess and quantify the extent of the SLGN problem (prominence, population densities and frequency of occurrence) and ii) determine which SLGN as well as grass species are involved by means of morphological (SLGN as well as grasses) and molecular identification (only SLGN).

3. Materials and methods

3.1. Nematode survey

Personnel of the seed Advance Seed collected *Eragrostis* spp. seed samples from 13 localities in the major production areas of South Africa and submitted them to the Nematology Unit of the ARC-Grain Crops Institute, Potchefstroom. These samples were stored in the laboratory at approximately 25°C. Within 7 days of receipt 1g SLGN-infected seed samples from each of the 13 localities were soaked in tap water in Petri dishes for 48 hours at 25°C to facilitate extraction of the nematodes. SLGN were counted under a dissection microscope and population density, frequency of occurrence (%) and Prominence values (PV) for each nematode population were calculated (Norton, 1978) as follows:

(i) Population density = $\frac{\text{total number of a nematode species / genera present}}{\text{number of plots on which the nematode species occurred}}$

(ii) Frequency of occurrence =

$$\frac{\text{number of localities where the nematode species / genus occurred}}{\text{number of localities sampled}} \times 100$$

(iii) PV = population density x $\sqrt{\text{frequency of occurrence}}$ / 10

3.2. Identification of SLGN individuals

3.2.1 Morphological identification

De Grisse's method as presented by Hooper *et al.* (2005) was used to prepare the SLGN for identification and are summarised below. SLGN were killed and fixed in 50ml tap water by adding the same volume of hot 8-% formaldehyde (50ml) to the SLGN sample, bringing the final concentration of formaldehyde to 4 %. It is important to use hot fixative because cool fixative often results in distortion of the tissues of nematodes (Hooper *et al.*, 2005). The SLGN specimens were then transferred from the fixative to small dishes (6.5cm diameter) containing glycerin-I solution. The glycerin-I solution consisted of 99ml of a 4-% formaldehyde solution and 1ml glycerin. The small dishes containing nematode specimens suspended in the glycerin-I solution were then placed in a desiccator that contained approximately 200ml 96-% ethanol solution to saturate the atmosphere. The desiccator containing the nematodes was placed in an oven at 30°C and left overnight (± 12 hours). The formaldehyde solution replaced the alcohol solution during this latter period. The small dishes containing the nematodes were subsequently removed from the desiccator and placed in an oven at 30°C. Five to 10ml of glycerin-II solution was then added to these dishes every one or two hours for a 24-hour period. The glycerin-II solution consisted of 95ml of a 6-% ethanol and 5ml glycerin solution. During this period in the oven the ethanol evaporated slowly and the nematodes remained immersed in pure glycerin.

Before the nematodes were transferred to a microscope slide a paraffin wax ring was stamped onto each slide using a device as shown in Fig 2.1 of which the one end was heated in a flame before pressing it into the paraffin wax. The nematodes were transferred individually to a drop of pure glycerin placed in the middle of each paraffin wax ring. After that a cover glass that fitted over the wax ring was placed over it and heated on a hot plate until the wax melted. The slide was removed from the hot plate until the wax had cooled down. Acrylic nail hardener (Cutex) was used to seal each cover glass around the edge and each mount was left to dry before studying the specimens for identification purposes under a light microscope at 200x

magnification. Slides containing mounted nematode specimens were then also sent to Dr. Antoinette Swart[2] to confirm the morphological identifications.

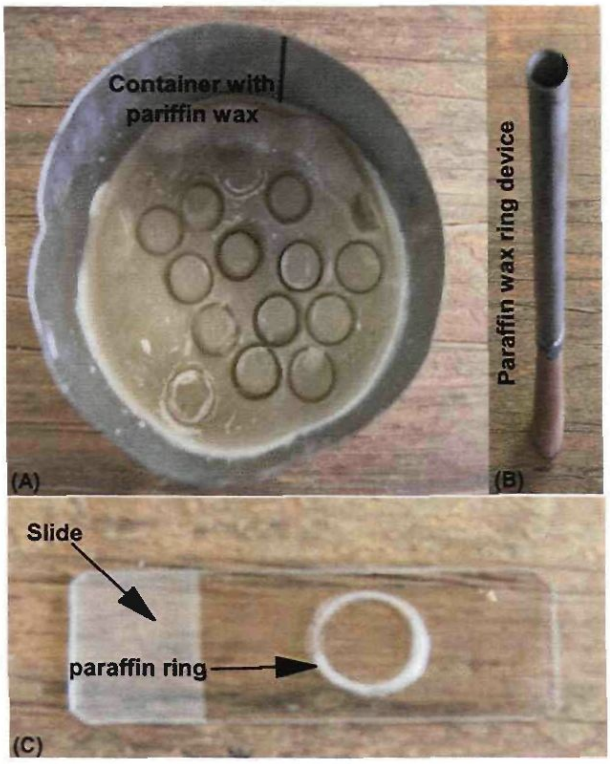

Figure 2.1. Illustration of (A) the paraffin wax used during this study as well as (B) the paraffin wax ring device used to stamp the paraffin wax ring onto (C) the microscope slides for morphological identification of seed- and leaf-gall nematodes (SLGN).

3.2.2. Molecular identification

SLGN specimens were also identified according to molecular techniques with the assistance of Dr. Charlotte Mienie (ARC-GCI[3]). These techniques are highly sensitive and compared to morphological identification facilitate more accurate identification of nematode species (Zijlstra *et al.*, 2000).

[3] Dr. Mienie, C.M., Department of Biotechnology Plant Breeding, ARC-GCI, Private Bag X1251, Potchefstroom, 2520, South Africa.

3.2.2.1 Extraction of DNA

For DNA extraction 25 nematodes were individually collected from nematode suspension extracted from each of the 13 localities from which we received *Eragrostis* seed. The 25 nematodes from each locality were placed in Eppendorf tubes containing 5µl of double-distilled water. Additionally 25 SLGN J2 and J3 from a reference population of *A. agrostis* supplied by Subbotin[4] as well as a reference population of *S. wevelli* supplied by GCI were also prepared as those from the 13 localities. A dissection microscope was finally used to check whether each Eppendorf tube contained 25 SLGN.

The DNA-extraction method used in this study was described by Stanton *et al.* (1998). DNA was extracted by adding 20µl of a 0.25-M NaOH solution to each Eppendorf tube with 25 nematodes. The tubes were centrifuged in a Grant Bio's centrifuge/vortexer combi-spin PCV-2000 at 3 000rpm for 30 seconds and then placed in a water bath at a temperature of 25°C and incubated overnight (± 12 hours). The tubes were then removed, centrifuged for a short period (±30 seconds at 3 000rpm) and heated to 94°C for 3 minutes in a thermal block. Subsequently the tubes were left to cool down to room temperature before they were centrifuged again for 30 seconds at 3 000g to force the nematode DNA to the bottom of each of the Eppendorf tubes. To each of the tubes the following solutions were then added: (i) 4µl of a 1-M HCl solution, (ii) 5µl of a 1-M Tris-HCl solution (pH 8.0) and (iii) 5µl of a 2-% Triton X-100 solution. The tubes were centrifuged again for 30 seconds at 3 000g and heated to 94°C for 3 minutes in the thermal block. After a cooling down period the tubes were centrifuged (30 seconds for 3 000rpm) for the last time to ensure that all the liquid containing the DNA was concentrated at the bottom of the respective tubes. The tubes were subsequently placed in a –20°C freezer and left overnight prior to the commencement of the polymerase chain reaction (PCR) procedure.

[4] Dr. Sergei Subbotin, Associate Nematologist, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento CA 95832, USA.

3.2.2.2 Polymerase chain reaction (PCR)

One set of PPN nematode primers was used for the PCR reaction to amplify the ITS1 region of the nematode DNA. The primer set consisted of a forward primer (TW81) and a reverse primer (AB28) (Zeng *et al.*, 2000; Swart *et al.*, 2004; Hooper *et al.*, 2005). PCR was conducted using 1µl of nematode DNA from each of the 13 localities suspended in a total volume of 25µl for each tube, which contained 5pmol of each of the forward (TW81) and reverse (AB28) primers as well as Promega PCR Master Mix (0.5µl Taq DNA polymerase, proprietary reaction buffer (pH8.5), 200µM each dNTP, 1.5mM $MgCl_2$).

PCR cycling was done in a MBS Satellite 0.2G Thermal cycler system (Thermo Hybaid) with one cycle at 94°C for 2 minutes, followed by 35 cycles at 94°C for 1min, at 55°C for 1min and at 72°C 1min, respectively. A final PCR cycling step of nematode DNA at 72°C for 5 minutes completed elongation of all PCR products for the 15 SLGN populations including the two reference populations. PCR products of these nematode populations were then analysed on a 2% agarose gel and visualised with ethidium bromide. Electrophoresis was carried out at 80V for 30 minutes. Photographs of DNA bands of the 15 nematode populations were produced under UV light using Quantity-1 software from Bio-Rad.

3.2.2.3 DNA sequencing

Before the nematode DNA of each of the 15 populations could be sequenced, it had to be purified and concentrated. The DNA clean and concentrator-5™ kit of Zymo Research (Catalogue Nos. D4003, D4004, D4013 and D4014) was used for the purification and concentrating of the nematode DNA. The purified DNA of each nematode population was then used for sequencing.

The DNA concentrations of the 15 nematode populations were determined using a spectrophotometer (Nanodrop, ND-1000). Sequencing was conducted using 2µl DNA from each population in a total volume of 20µl, containing 3µl 5x sequencing buffer (Applied Biosystems), 1µl TW81 primer,

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2µl terminator ready-mix and 12µl nuclease-free water. This latter procedure was also repeated for the reverse primer AB28. The sequence cycle was done in a MBS system (Thermo Hybaid) with one cycle at 96°C for 1 minute, followed by 25 cycles at 96°C for 10 seconds, at 50°C for 5 seconds, at 60°C for 4 minutes and held at 4°C. Subsequently the sequencing reaction mix for each of the 13 nematode populations was added to a mixture containing 50µl 100-% EtOH, 2µl 125-mM EDTA and 2µl 3M-NaAc, vortexed gently for ± 5-10 seconds and refrigerated for 20 minutes at –20°C. The samples were then spun at 10 000rpm for 20 minutes after which the supernatant was carefully removed with a micropipette without touching the transparent DNA pellet that was located at the bottom of each of the tubes. Subsequently the samples were washed with 120µl 100-% EtOH, vortexed for 5s and spun for 15 minutes at 10 000rpm. The supernatant was removed and the washing and centrifuging of the DNA repeated. The 15 samples were transferred and dried in a laminar flow cabinet in darkness for 20 minutes. When the samples were dry the DNA pellets were each individually resuspended in 10µl HiDi (supplied by Applied Biosystems). They were then placed in a sequencer (3130xl Genetic Analyzer: Applied Biosystems) under the following conditions, namely oven temperature of 60°C, injection voltage of 1.2 kVolts, injection time of 18 seconds and a running voltage of 8.5 kVolts.

After sequencing of the DNA, an electrophoretogram data of the nematodes was generated and edited by visual assessment to remove ambiguous nucleotides. The sequences for the 13 respective populations were then compared to sequences of the two reference populations as well as a *A. agrostis* sequence available in GenBank (www.ncbi.nlm.nih.gov). Multiple alignments were performed with ClustalX (Thompson *et al.*, 1997) using the default settings of gap-opening penalty of 15 and gap-extension penalty of 6. The 3' and 5' ends of all sequences were then trimmed to ensure that sequences of similar lengths were used for each alignment. ClustalX was used to generate DNA distance matrices between the individual nematode populations as well as the reference populations. Dendrograms were constructed using the neighbour-joining (NJ) method. The reliability of the branches of each tree was verified with bootstrap tests of 100 replications run

for each entry. The software programme TreeView (Win 32, ver. 1.6.6) was used to compile dendograms to determine the relationship between the SLGN populations.

4. Results

4.1. Survey

Table 2.1: Seed- and leaf-gall nematode (SLGN) numbers/1g seed, population density, frequency of occurrence (%) and prominence values (PV) from 13 localities representative of *Eragrostis* spp. grass-producing areas of South Africa.

Locality	SLGN numbers/1g seed	SLGN population density and PV	SLGN frequency of occurrence (%)
1. Amersfoort 5	2 668 (e)*	205	100
2. Potchefstroom	2 483 (e)	191	100
3. Amersfoort 6	1 018 (de)	78	100
4. Amersfoort 3	723 (de)	56	100
5. Amersfoort 2	280 (cd)	22	100
6. Amersfoort 1	173 (d)	13	100
7. Amersfoort 7	110 (bc)	9	100
8. Amersfoort 9	95 (ab)	7	100
9. Amersfoort 8	71 (b)	5	100
10. Oordrag 2	29 (ab)	2	100
11. Oordrag 1	22 (ab)	2	100
12. Amersfoort 4	2 (a)	0.2	100
13. Bethal	1 (a)	0.1	100

* Means in the same column followed by the same letter do not differ significantly ($P \leq 0.05$) according to Tukey test.

Results from the survey indicated that seed from all 13 localities that were sampled were infected with SLGN (Table 2.1). Substantial variation existed among the localities with regard to SLGN population levels, with numbers ranging from 1 (Bethal) to 2 668/1g of seed (Amersfoort 5). Amersfoort 5, followed by Potchefstroom maintained the highest SLGN numbers. Population densities and PV values for the SLGN sampled at the 13 sites ranged between 0.1 for Bethal and 205 for Amersfoort 5. Localities

Amersfoort 4 and Bethal maintained the lowest SLGN numbers, population densities and PV.

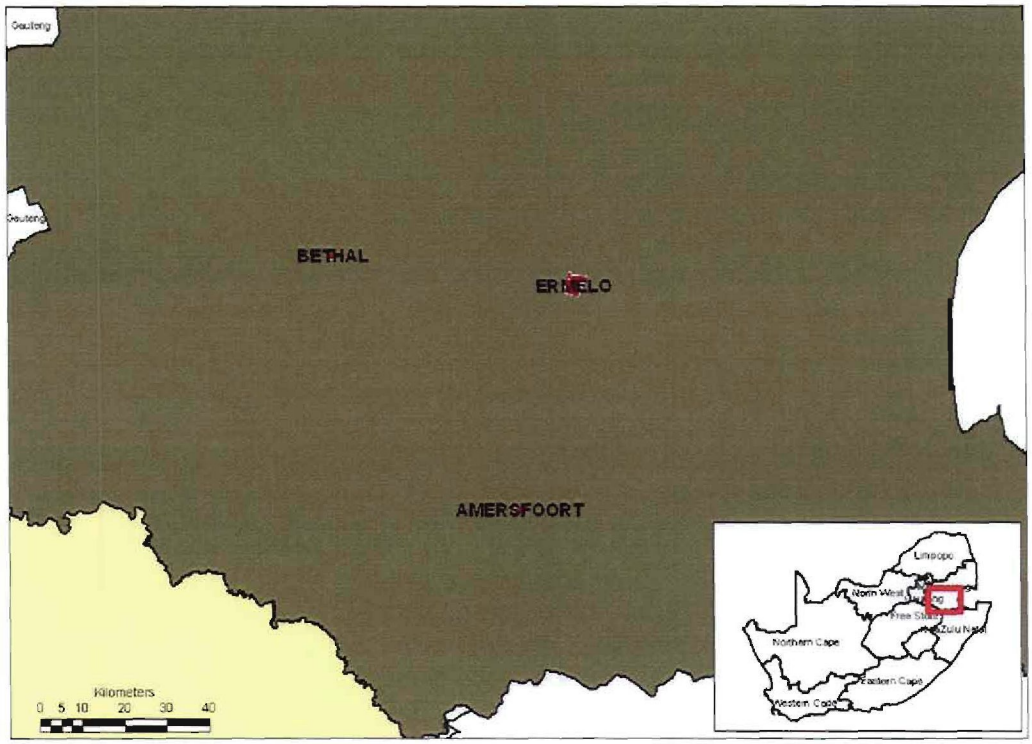

Figure 2.2. A map showing the Bethal-Ermelo-Amersfoort area in the Mpumalanga Province where *Eragrostis curvula* seed were collected from farms of 13 producers to determine the occurrence and spread of the seed- and leaf-gall nematode (SLGN).

4.2 Morphological identification

Presence of second- (J2) and third (J3)-stage juveniles only of SLGN in seed samples obtained during the survey hampered morphological identification to species level. Mature males and females are essential for accurate species identification of SLGN reference (Heyns, 2006). J2 and J3 from all 13 localities could, however, be identified to genus level as being *Anguina/Subanguina* on account of their morphological characteristics (Van den Berg, 1985; Kleynhans *et al.*, 1996).

4.3 Molecular identification

4.3.1 PCR of the *Anguina/Subanguina* spp. from the 13 different localities sampled during the survey.

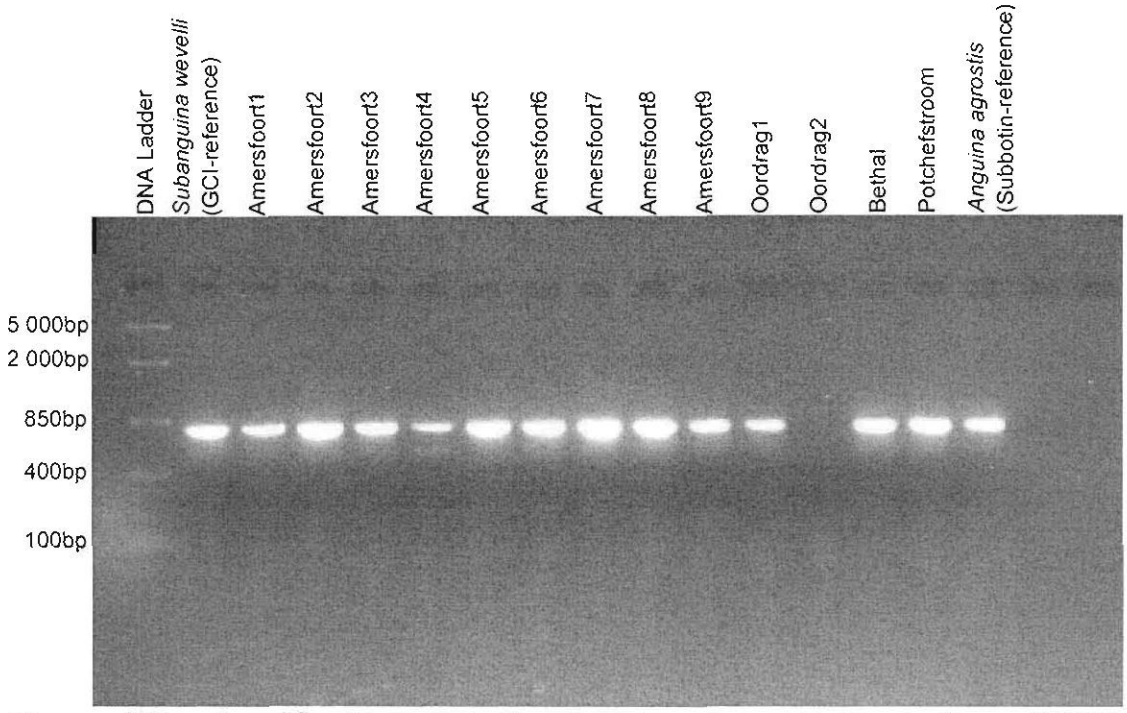

Figure 2.3. Amplification products of polymerase chain reactions (PCR) illustrating the seed- and leaf-gall nematode (SLGN) DNA (white bands) from the 13 localities as well as those for the *Subanguina wevelli* (GCI-reference) and *Anguina agrostis* (Subbotin-reference) using forward (TW81) and reverse (AB28) primers.

No pronounced differences were observed in the location of the DNA bands of SLGN from 12 of the 13 localities, as well as between the latter and the two reference populations (Fig. 2.3). PCR with both the forward and reverse primers resulted in a fragment of approximately 850bp being amplified for all the nematode DNA evaluated. DNA extracted from SLGN infecting *Eragrostis* spp. seed at Oordrag 2, however, was inadequate and of poor quality and therefore no fragment could be amplified.

4.3.2 Sequencing of SLGN DNA

Bootstrap values (Fig. 2.4) for the various SLGN populations varied substantially. They ranged from 2 for AB28 *S. wevelli* (GCI-reference) to 100 for TW81 *A. agrostis* (Subbotin-reference). All SLGN populations with bootstrap values smaller than 90% did, however, not differ significantly from the other SLGN populations clustered together with *S. wevelli* (GCI-reference) when both primers were used. A significant difference could be distinguished between the *S. wevelli* cluster and those of both references for *A. agrostis* (GenBank and Subbotin). However, *A. agrostis* TW81 differed significantly from the *A. agrostis* AB28 and the *A. agrostis* from GenBank. The similarity values (%) calculated between the ITS regions of the various *S. wevelli* found at the 12 different localities apart from Oordrag 2 as well as those of the reference populations varied between 98% to 100%, resulting in only a 2% difference between isolates (see Table 1 in Appendix A). On the other hand, these similarity values ranged from 84% to 86% when calculated for the *S. wevelli* populations and *A. agrostis* populations (GenBank and Subbotin), implying a difference of more than 10%.

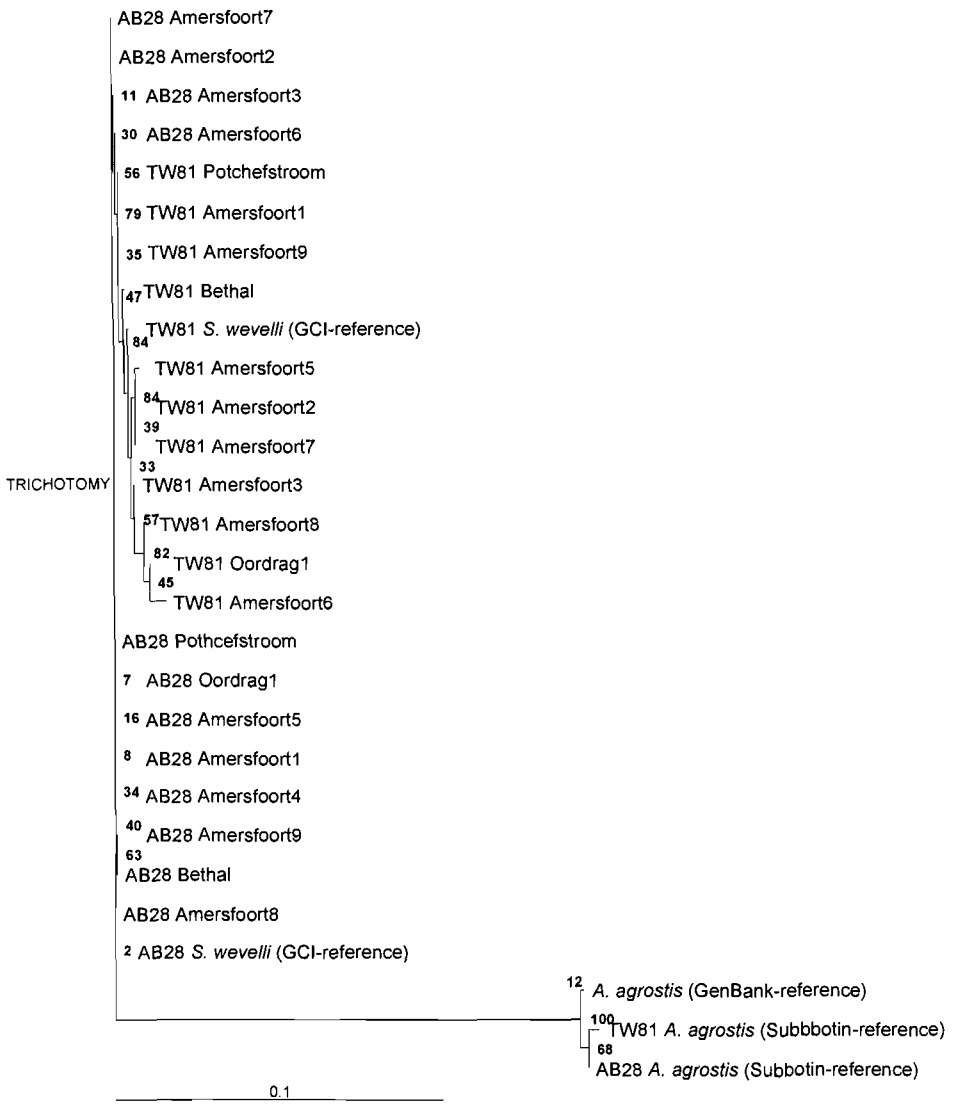

Figure 2.4. Neighbour-joining (NJ) tree based on the internal-transcribed spacer (ITS) region of sequenced SLGN isolate bootstrap values, inter- and intraspecific relationships between SLGN from 12 localities as well as reference populations of *Subanguina wevelli* (GCI-reference) and *Anguina agrostis* (GenBank- and Subbotin-reference).

5. Discussion

SLGN were present in seed of *Eragrostis curvula* at all 13 localities sampled during the survey. Although the PV of these SLGN varied from low to relatively high, the 100% frequency of occurrence of SLGN at all localities supports and confirms the threat this parasite poses to grass seed producers and related industries. These results confirm those by Fourie and Mc Donald (2002), who reported relatively high numbers (2 094/3g) of SLGN in *E. curvula* seed from Ermelo, which is not far from Amersfoort. Merely the presence of *S. wevelli*, despite their population levels, in *E. curvula* seed consignments renders these parasites a serious problem to South African producers since infected seed is rejected by other world countries according to legislation (personal communication, Mr. Tony Sibert, Advance Seed[1] and Dr. A. Swart, ARC-PPRI[2]).

Although morphological identification of SLGN from the 13 localities sampled during this survey could only confirm the genus as either being *Anguina* or *Subanguina*, results of molecular techniques used provided positive confirmation that at least 12 of the 13 populations sampled belong to the species *S. wevelli*.

This is substantiated by phylogenetic tree data, since these 12 SLGN populations had bootstrap values lower than 90%. Therefore, they did not differ significantly (Subbotin & Moens, 2006) from each other or the *S. wevelli* reference (GCI) used. Consolidation of the identification of *S. wevelli* from the 12 localities were also proved by low similarity values obtained between the ITS regions of these populations. The phylogenetic tree constructed from the DNA sequence data of 12 of the 13 SLGN populations in this study was in agreement with those of Subbotin *et al.* (2004) in terms of the placement of these local *S. wevelli* populations. When the phylogeny of a PPN is estimated it should be accompanied by an indication of its confidence limit (Subbotin & Moens, 2006). Since bootstrapping is the more common and accurate approach used to estimate the confidence levels of branches in phylogenetic trees (Subbotin & Moens, 2006), they were also used as the criteria for placement of the local *S. wevelli* populations sampled in this study.

Both the *A. agrostis* references included in this study clustered on a different branch than the local *S. wevelli* populations, indicating that the former belong to related, but different genera of SLGN. The significant difference between the two *A. agrostis* references is also in agreement with results from Subbotin *et al.* (2004) and could be explained as being from different populations sourced from different geographical areas. The results of this study are further substantiated by Powers *et al.* (2001), who confirmed through ITS1 nucleotide sequencing that *S. wevelli* is a separate species and is grouped separately from species such as *A. funesta* and *A. agrostis*.

Since localities Amersfoort 5 and Potchefstroom maintained the highest numbers of SLGN and therefore had the highest infection per seed sample, seed from these two localities were used for further trails throughout this study. Since this nematode species is seed-borne it is crucial to investigate potential strategies that may provide economically acceptable and efficient ways for controlling this parasite in the short and long term.

6. Conclusions

Ultimate identification of SLGN species on the basis of morphological identification only is not possible when juveniles only are present in such populations, which was the case during this study. It is thus agreed with Riley *et al.* (1988) that more sensitive and reliable techniques such as molecular, are required for distinguishing between *Anguina* species. *Subanguina wevelli* populations from the localities sampled are, therefore, reared in vivo on *E. curvula to* obtain mature males and females in order to also confirm morphological identification of these species.

CHAPTER 3: Evaluation of mechanical, commercialisable, control methods for the reduction of seed- and leaf-gall nematode numbers in *Eragrostis* spp. seed.

1. Introduction

Although acceptable and efficient control options are available for the management of *Anguina tritici* on wheat, limited information is available on the control of *Subanguina wevelli* on *Eragrostis curvula* (weeping lovegrass) under local environmental conditions. One of the potentially acceptable options to avoid *S. wevelli* infection is to move Eragrostis seed production away from areas where moist environmental conditions prevail, e.g. early-morning dew prevailing in the mist belt of South Africa. Chemical control will be discussed in Chapter 4 as another option.

The ultimate objective is production of SLGN-free seed because the nematode is currently by no means under control. Since SLGN are seed-borne, it is crucial to investigate potential strategies that will provide economically viable and efficient control of these parasites over the short as well as long term. Therefore, the development of control strategies focussed on SLGN in Eragrostis seed needs to be investigated in order to address this problem in a more sustainable manner in this grass.

2. Specific objective

The objective of this study was to investigate mechanical, commercialisable techniques for separating SLGN-galled Eragrostis seed from uninfected seed.

Figure 3.1. *Eragrostis curvula* (Ermelo variety) seed containing dark- coloured, galled seed (circled in white) infected with seed- and leaf-gall nematodes (SLGN).

3. Materials and methods

Due to limited availability of Eragrostis seed, only 0.5g samples could be allocated to each treatment for both the i) sieving and ii) floating- and brine sedimentation trials. Subsequently for each of the 12 and 6 replicates used for the i) sieving and ii) float and brine trials, respectively, 0.5g of seed were used per treatment.

3.1 Sieving of seeds

Eragrostis seed was randomly harvested at Amersfoort 5 production areas. Seed batches of 0.5g each from this source were passed through three mesh sieves 600-µm, 500-µm and 250-µm apertures. The control treatment was not passed through the sieves. The trial was replicated 12 times. The number of galled SLGN seeds on each sieve was determined by means of manual counting. Seed mass per sieve was determined. Samples from each sieve were then soaked in ± 20ml tap water and incubated in a growth cabinet for 48 hours at 25°C for extraction of SLGN. The extracted nematodes were collected by pouring the nematode suspension through a 25-µm-mesh sieve

and washing them into a 20ml sample bottle. Nematodes were counted using a dissection microscope.

In addition to this, 20 seed in total constituting both SLGN-galled and uninfected seed were randomly collected from each sieve and subjected to germination tests in order to establish the effect of seed size (determined by different sieves) on seed viability. For germination, seeds were covered in damp tissue paper and left at 25°C in a growth chamber for 3 days. The number of germinated seeds for each were then counted and expressed as percentage germination. Nematode data were ln(x+1) transformed and subjected to an analysis of variance (ANOVA) (Statgraphics Plus 5 for Windows). The ANOVA was based on four treatments, viz. the three sieves plus the un-sieved control, each replicated 12 times.

3.2 Floating- and brine sedimentation

Samples of 0.5g of Eragrostis seed from the same source as for the sieving trial (section 3.1) were soaked in a range of 0 (untreated control), 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5% and 20% NaCl solutions in tap water for 5 min. The above-mentioned untreated control comprised of tap water only and each treatment was replicated six times. The total number of seeds and the number of SLGN galls present in each 0.5g sample were counted before the trial commenced. After the immersion in the solutions the number of seeds and galls that floated as well as those that sank after 5 min of soaking were counted for each treatment to determine the respective percentages. Both the seed and galls that floated as well as those that sank for each treatment were subsequently removed manually with a 250-µm-mesh sieve and soaked in a Petri dish (6.5cm diameter) in ± 20ml tap water. The Petri dishes containing the seed and galls were then incubated in a growth cabinet for 48h at 25°C. SLGN specimens extracted after this period were then poured on a 25-µm-mesh sieve and washed into 20ml sample bottles with tap water. Nematodes were counted under a dissection microscope.

In total twenty-five randomly collected seeds, which included both SLGN-galled and uninfected seed from each treatment, were collected after it were

subjected to the NaCl. These seed were subjected to germination tests in order to establish the effect of the NaCl treatments on seed viability. Germination was done as described in section 3.1. Nematode data were ln(x+1) transformed before subjected to an ANOVA.

3.3 Sieving of seed combined with floating- and brine sedimentation

In order to optimise the efficacy of mechanical separation of *S. wevelli*-infected galls from uninfected Eragrostis seed, the sieving and NaCl techniques were combined.

For the sieving trial three treatments consisting of three mesh sieves 600-µm, 500-µm and 250-µm apertures were used. The untreated (unsieved) control treatment was not subjected to sieving. Each of the treatments was replicated five times with 400g-seed batches of Eragrostis seed infected with *S. wevelli* collected from the Standerton area (Mpumalanga Province). Sieving was done as described in section 3.1. The seed that remained behind on each of the three sieves was equally divided into two seed batches after it was weighed. One of these batches for each treatment was the untreated control (only tap water), while the other seed batch for each treatment was subjected to a 15% NaCl solution for 5 minutes as indicated in section 3.2. The number of replicates remained five as for the preceding sieving trial. Subsequently both floating and sunken seed were removed from both the untreated control and the NaCl treatments using a 25-µm-mesh sieve. Nematodes were then extracted from floating and sunken seed by soaking these seed samples in 500ml tap water for 48h at 25°C. SLGN specimens extracted after this period were poured on a 25-µm-mesh sieve and washed into 20ml sample bottles using tap water. Nematodes were counted using a dissection microscope. SLGN data were ln(x+1) transformed and subjected to an analysis of variance (Statgraphics Plus 5 for Windows).

4. Results

4.1 Sieving of seed

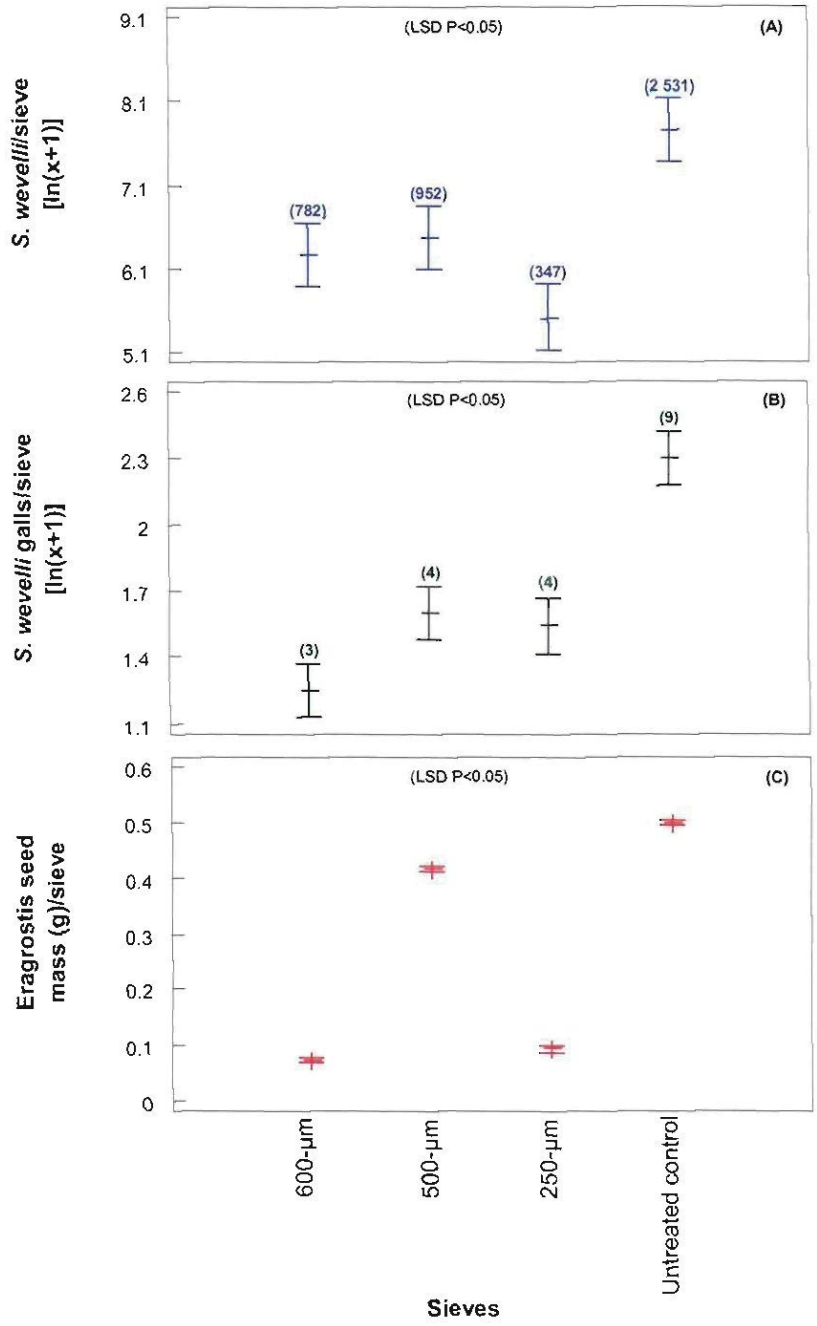

Figure 3.2. Number of (A) *Subanguina wevelli* extracted, (B) galls collected and (C) *Eragrostis curvula* seed mass for the three sieve treatments as well as the untreated control. (Mean nematode numbers (A) as well as galls (B) are indicated in parenthesis).

Table 3.1: F-ratios and P-values of the number of *Subanguina wevelli*, galls and *Eragrostis curvula* seed mass for each of the four treatments.

Main effects	*S. wevelli*/sieve [ln(x+1)]		*S. wevelli* galls/sieve [ln(x+1)]		Eragrostis seed mass/sieve	
	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value
B: Treatment	12.10	0.0000	26.62	0.0000	2969.32	0.0000
C: Replicate	1.84	0.0863	0.54	0.8581	0.38	0.9563

4.1.1 Numbers of *S. wevelli*

The mean number of nematodes extracted from seed collected from each of the sieves ranged from 347 for the 250-µm-mesh sieve to 952 on the 500-µm-mesh sieve, while the untreated control had 2 531 nematodes (Fig. 3.2A). The 250-µm-mesh sieve maintained the lowest number of *S. wevelli* and differed significantly from the 500-µm-mesh sieve and the untreated control, but not from the 600-µm-mesh sieve (Fig. 3.2A; Table 3.1). The untreated control maintained the highest *S. wevelli* numbers and differed significantly from all three sieve treatments.

The 250-µm-mesh sieve contained 86.3%, the 500-µm-mesh sieve 62.4% and the 600-µm-mesh sieve 69% fewer *S. wevelli* in seed compared to the untreated control.

4.1.2 Numbers of *S. wevelli* galls

The mean number of *S. wevelli* galls on the three sieve treatments ranged between 3 for the 600-µm-mesh sieve and 4 for the 250-µm-mesh and 500-µm-mesh. The untreated control had 9 galls, which was the highest (Fig. 3.2B). The number of galls in the untreated control differed significantly with the 600-µm-mesh sieve, which had the lowest number of galls (Fig. 3.2B; Table 3.1). The 500-µm- and 250-µm-mesh sieves did not differ significant from one another.

The 250-µm- and 500-µm-mesh sieves contained 56%, while the 600-µm-mesh sieve contained 67% fewer galls compared to the untreated control (data not shown). The mean number of nematodes per gall was the lowest for the 250-µm-mesh sieve (87 nematodes/gall) and highest for the untreated

control (281), while the means for the 600-µm- and 500-µm-mesh sieves were 261 and 238 nematodes/gall, respectively.

4.1.3 Seed mass

The seed mass for each of the four treatments ranged between 0.07g on the 600-µm-mesh and 0.4g for the 500-µm-mesh sieve. The 0.5g of the untreated control remained intact since they were not subjected to the sieving method (Fig. 3.2C). A significant difference existed between the four treatments, with the seed mass on the 500-µm-mesh sieve the highest but still significantly less than the untreated control. The 600-µm-mesh sieve had the lowest seed mass, which was significantly less than the other two sieves and the untreated control. The 250-µm-mesh sieve had significantly less seed mass than the 500-µm-mesh sieve and the untreated control. Except for the untreated control that was highest with respect to all variables the seed mass on the three sieves did not correspond to their respective nematode or gall numbers (Fig. 3.2C; Table 3.1).

Data of the germination tests showed that 83% of the seed was still viable for germination, with the 500-µm-mesh sieve having the highest geminating percentage between the three sieve treatments (Table 3.2).

Table 3.2: The percentage Eragrostis seed that germinated after it was subjected to the sieving method.

Treatment	**Population 1**
	***E. curvula* seed that germinated (%)**
1. 600-µm	84
2. 500-µm	86
3. 250-µm	81
4. Untreated control	80

4.2 Floating- and brine sedimentation

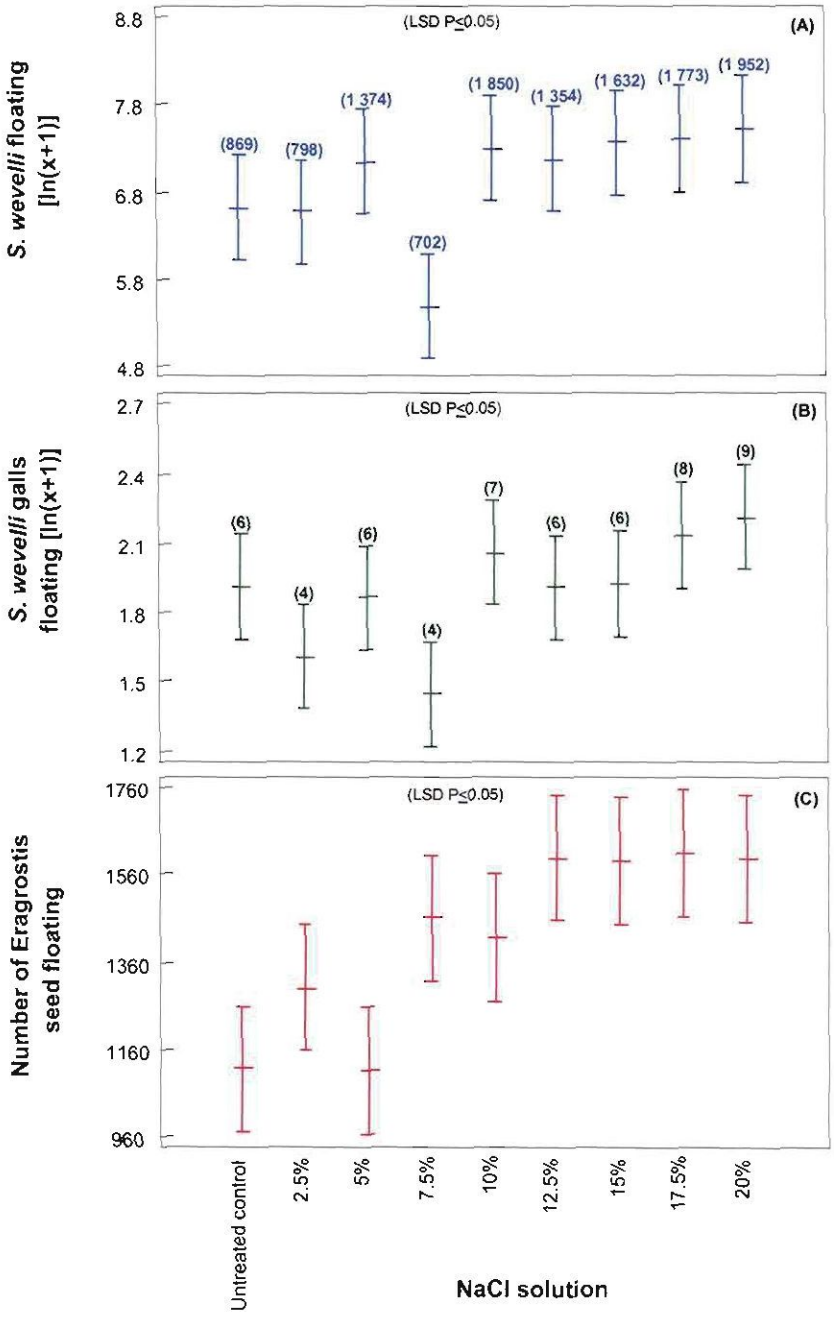

Figure 3.3. Number of (A) *Subanguina wevelli* extracted and (B) galls collected as well as the (C) number of Eragrostis seed from the floating seed for the nine treatments. (Mean nematode (A) and gall (B) numbers are given in parenthesis).

Table 3.3: F-ratios and P-values for the *Subanguina wevelli* population with regard to the number of juveniles, galls and Eragrostis seed that floated for each of the nine treatments.

Main effects	*S. wevelli* floating [ln(x+1)]		*S. wevelli* galls floating [ln(x+1)]		Number of Eragrostis seed floating	
	F-ratio	P-values	F-ratio	P-values	F-ratio	P-values
Treatment	2.32	0.0375	2.31	0.0386	4.05	0.0013
Replicate	0.89	0.4967	0.68	0.6385	0.91	0.4864

4.2.1 Numbers of *Subanguina wevelli* extracted from floating seed

The mean number of nematodes extracted from Eragrostis seed that floated after it was immersed in the NaCl treatments was relatively high. It ranged from 702 for 7.5% NaCl to 1 952 for the 20% treatment (Fig. 3.3A). The untreated control did not differ significantly from the NaCl treatments (Fig. 3.3A; Table 3.3). Although the 7.5% NaCl resulted in the lowest SLGN numbers and differed significantly from the 5%, 10%, 12.5%, 15%, 17.5% and 20% treatments, it did not differ significantly from the untreated control and the 2.5% treatment.

4.2.2 Numbers of *Subanguina wevelli* galls that floated

The mean number of nematode galls was relatively low for all the treatments. It ranged from 4 for both the 2.5% and 7.5% NaCl to 9 for the 20% NaCl (Fig. 3.3B). Although the 7.5% NaCl maintained the lowest number of *S. wevelli* galls, it did not differ significantly from the 2.5% and 5% treatments. However, the 7.5% treatment had significantly fewer *S. wevelli* galls than the untreated control, 10%, 12.5%, 15%, 17.5% and 20% NaCl. The 17.5% and 20% treatments resulted in the highest number of SLGN galls and only differed significantly from the 2.5% and 7.5% treatments (Fig. 3.3B; Table 3.3).

4.2.3 Number of Eragrostis seed that remained afloat

Significant differences existed among the treatments with regard to seed that floated after subjection to the different NaCl solutions (Fig. 3.3C; Table 3.3). The 5% treatment had the lowest number of seed that floated, but did not differ significantly from the untreated control and the 2.5% treatment. The

2.5% treatment did, however, only differ significantly from the 12.5%, 15%, 17.5% and 20% treatments, while the 5% NaCl differed significantly from the 7.5% treatment and higher. The majority of Eragrostis seed floated after submersion in the different NaCl solutions. The percentage E. curvula seed that remained afloat for the nine NaCl-treatments ranged between 55% for the 5% NaCl and 80% for the 20% NaCl treatment (Table 3.4). The mean number of nematodes per gall in this trial ranged between 176 to 264 (data not shown).

Data from germination tests showed that a mean of 70% of the *E. curvula* seed that remained afloat were still viable for germination, with the 2.5% NaCl treatment maintaining the highest percentage germinating seed (Table 3.4).

Table 3.4: The percentage of Eragrostis seed that floated and germinated after being subjected to a range of NaCl-solution treatments for the *Subanguina wevelli* population.

NaCl treatment	**Percentage *E. curvula* seed floating**	***E. curvula* seed germinated (%)**
Untreated control	56	67
2.5 %	64	77
5 %	55	76
7.5 %	71	69
10 %	71	73
12.5 %	78	66
15 %	79	65
17.5 %	79	63
20 %	80	74

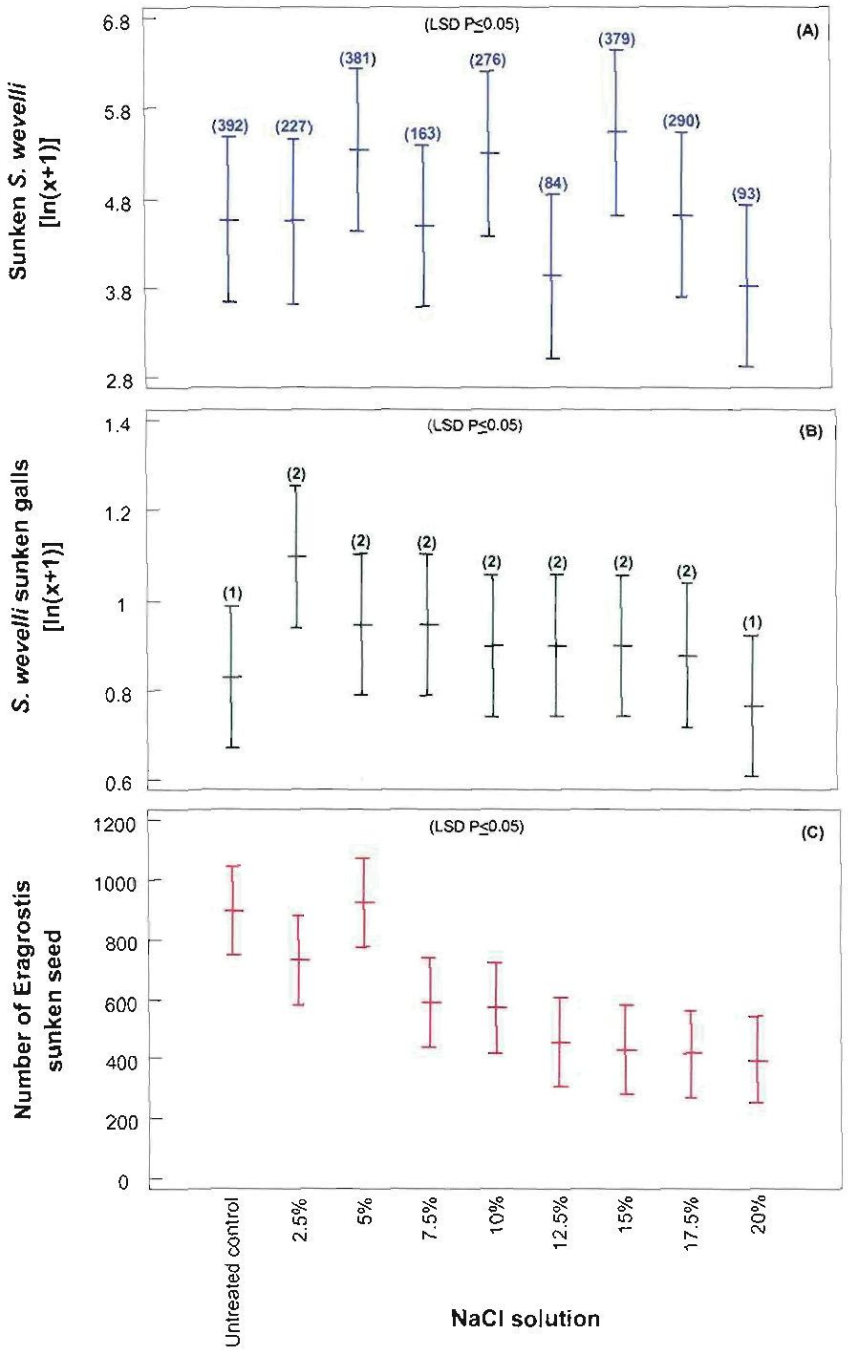

Figure 3.4. Number of (A) *Subanguina wevelli* juveniles extracted and (B) galls collected as well as the (C) number of *Eragrostis curvula* seed from the sunken seed for the nine treatments. (Mean nematode (A) and gall (B) numbers are given in parenthesis).

Table 3.5: F-ratios and P-values for the *Subanguina wevelli* population with regard to the number of juveniles, galls and *Eragrostis curvula* seed that sunk for each of the nine treatments.

Main effects	**Sunken *S. wevelli* [ln(x+1)]**		***S. wevelli* sunken galls [ln(x+1)]**		**Number of Eragrostis sunken seed**	
	F-ratio	**P-values**	**F-ratio**	**P-values**	**F-ratio**	**P-values**
Treatment	0.90	0.5296	0.68	0.7076	3.81	0.0021
Replicate	0.24	0.9422	0.25	0.9380	0.82	0.5458

4.2.4 Numbers of *Subanguina wevelli* extracted from sunken seed

The number of *S. wevelli* extracted from *E. curvula* seed that sunk after it was submerged in the eight NaCl treatments ranged between 84 for the 12.5% NaCl and 381 for the 5% treatment (Fig. 3.4A). The untreated control contained 392 *S. wevelli* in sunken seed. No significant differences existed between the nine treatments with regard to the number of SLGN that sunk in the experiment (Fig. 3.4A; Table 3.5).

4.2.5 Numbers of *Subanguina wevelli* galls that sunk

The number of *S. wevelli* galls that sunk was low and ranged between 1 for the 20% NaCl as well as the untreated control to 2 for all the other NaCl treatments (Fig. 3.4B). No significant differences were therefore recorded among the treatments (Fig. 3.4B; Table 3.5).

4.2.6 Number of Eragrostis seed that sunk

The untreated control, 2.5% and 5% NaCl solutions had the highest number of sunken seed and differed significantly to all the other treatments, except the 2.5% (Fig. 3.4C; Table 3.5). Although 20% NaCl maintained the lowest number of seed that have sunken, it did only differ significantly from the 2.5% and 5% treatments and the untreated control. The percentage Eragrostis seed that sunk ranged between 20% for the 20% NaCl and 45% for the 5% NaCl treatment (Table 3.6).

Data of the germination tests showed that 82% of the Eragrostis seed that sunk was still viable for germination, with the 17.5% and 20% NaCl treatments having the highest germination percentages (Table 3.6).

Table 3.6: The percentage of *Eragrostis curvula* seed that sunk and germinated after subjected to a range of NaCl-solution treatments for the *Subanguina wevelli* populations.

NaCl treatment	**Percentage *E. curvula* seed sunken**	***E. curvula* seed germinated (%)**
Untreated control	44	79
2.5 %	36	79
5 %	45	83
7.5 %	29	77
10 %	29	82
12.5 %	22	78
15 %	21	85
17.5 %	21	87
20 %	20	87

4.3 Sieving of seed combined with floating- and brine sedimentation

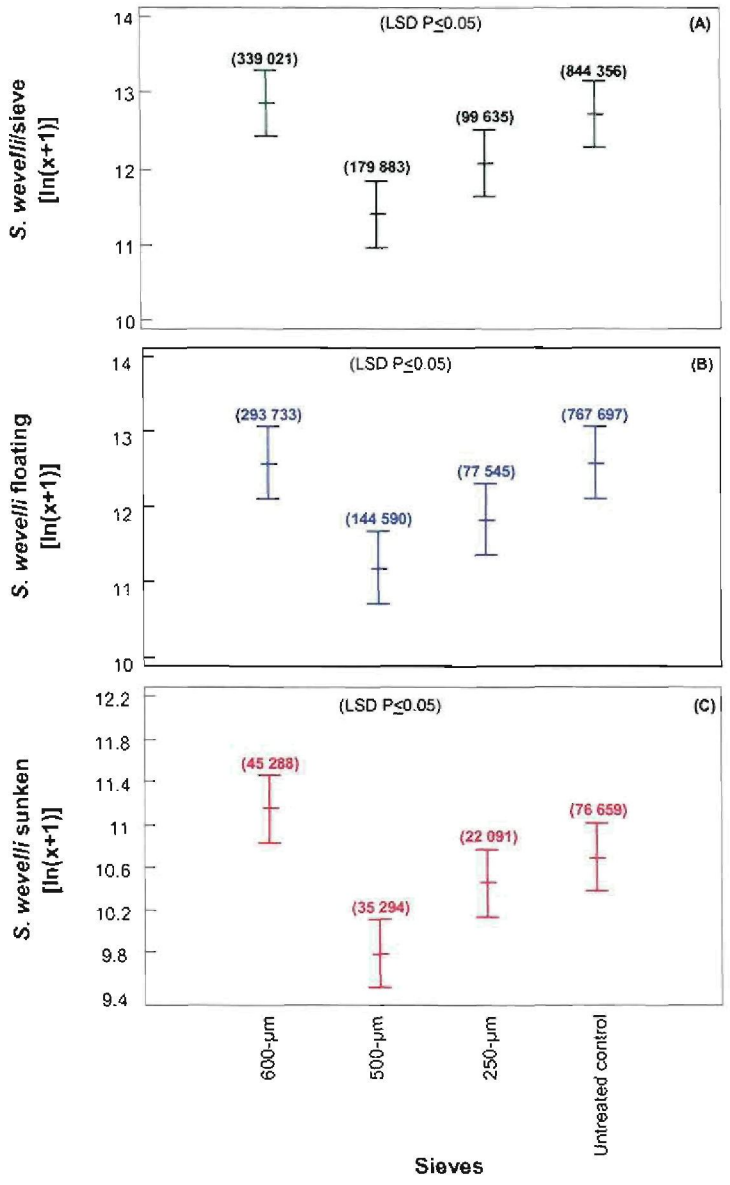

Figure 3.5. (A) *Subanguina wevelli* numbers extracted from Eragrostis seed contained by each of the three sieve treatments (600-µm, 500-µm and 250-µm) as well as the untreated control. (B) Number of nematodes that were extracted from the seed that remained afloat and (C) those that sunk after having been suspended in a 15% NaCl solution for 5 minutes. (Mean nematode numbers are indicated in parenthesis).

Table 3.7: F-ratios and P-values for *Subanguina wevelli* numbers with regard to the sieve treatments as well as the NaCl treatments.

Main effects	***S. wevelli* per sieve [ln(x+1)]**		***S. wevelli* floating [ln(x+1)]**		***S. wevelli* sunken [ln(x+1)]**	
	F-ratio	**P-value**	**F-ratio**	**P-value**	**F-ratio**	**P-value**
A: Treatment	5.44	0.0135	4.65	0.0223	7.41	0.0046
B: Replicate	1.71	0.2120	1.75	0.2046	1.12	0.3928

4.3.1 Number of *Subanguina wevelli* on the sieves

The mean number of *S. wevelli* that was retrieved during the sieving in this trial ranged between 99 635 for the 250-µm-mesh sieve and 339 021 for the 600-µm-mesh sieve (Fig. 3.5A). A significant difference existed between the three sieve treatments, with the 500-µm-mesh sieve containing significantly lower numbers of nematodes than the 600-µm-mesh sieve and the untreated control, but not the 250-µm-mesh sieve (Fig. 3.5A; Table 3.7). Only the 500-µm-mesh sieve contained significantly lower nematode numbers than the untreated control.

4.3.2 Number of *Subanguina wevelli* floating

The mean number of *S. wevelli* individuals that were extracted from seed that floated after it was submerged in a 15% NaCl solution was high. They ranged from 77 544 for the 250-µm-mesh sieve combined with the NaCl treatment to 293 733 for the 600-µm-mesh sieve and NaCl (Fig. 3.5B). The untreated control did not differ significantly from the 600-µm- and 250-µm-mesh sieves combined with NaCl, but it differed significantly from the 500-µm-mesh sieve NaCl (Fig. 3.5B; Table 3.7). The latter, however, did not differ significantly from the 600-µm-mesh sieve and NaCl.

4.3.3 Number of *Subanguina wevelli* sunken

The number of nematodes that were extracted from seed that sunk after it was submerged in a 15% NaCl solution was also high. They ranged from 22 091 for the 250-µm-mesh sieve to 45 288 for the 600-µm-mesh sieve (Fig. 3.5C). The untreated control did not differ significantly from the 600-µm and 250-µm-mesh sieves, but it did differ significantly from the 500-µm-mesh sieve (Fig. 3.5C; Table 3.7). The 500-µm-mesh sieve, however, contained

significantly fewer *S. wevelli* than the other two sieves. The latter two treatments did not differ significantly from each other and also not from the untreated control.

5. Discussion

Although sieving of Eragrostis seed provided a significant reduction in numbers of *S. wevelli* as well as number of galls the amount of both remaining in the seed defies the objective of the exercise. The success rate is comparable with others on other seed-borne nematode species (Courtney & Howell, 1952; Paruthi and Bhatti, 1992) but trade and export requirements disallow any contamination of seed batches[1].

Paruthi and Bhatti (1992) recorded complete removal of *A. tritici* galled wheat seeds with 10%, 15%, and 20% NaCl solution treatments. Results of this study, however, were not closely as successful in terms of removing *S. wevelli* galls from Eragrostis seed. Although substantially higher numbers of *S. wevelli* J2 and J3 were extracted from seed that floated after it was subjected to various concentrations of NaCl treatments, the number of nematodes in seed that sunk remained also unacceptably high. The same trend occurred with regard to SLGN-galled seed. Furthermore, the majority of seed subjected to sieving as well as to the NaCl treatments were floating. This implies that the biggest percentage of seed batches will be lost using this method.

Combination of the sieving, floating and brine sedimentation methods to minimize or totally remove *S. wevelli* from *E. curvula* seed also proved to be unsuccessful for the purpose of seed trade and export. Personal observation showed that the bigger the seed samples that were subjected to these mechanical separation methods in our study, the higher the residual number of *S. wevelli*, even for the treatments that were the most successful. This implies that use of these methods on a commercial scale for big amounts of seed will still render unacceptable high population levels of *S. wevelli*.

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[1] Mr. Tony Siebert, Advance Seed, 26 Collage Laan, Pothcefstroom, 2531, South Africa.

6. Conclusions

Mechanical separation of *E. curvula* seed by means of sieving as well as by NaCl sedimentation proved not to be effective in eliminating *S. wevelli* from seed, either when used as separate strategies or in combination. Planting of sieved seed as well as seed subjected to NaCl sedimentation will still lead to infection of Eragrostis in grass producing areas of South Africa. It is, therefore, crucial to investigate more effective means of separating SLGN galled seeds from uninfected seed and to use these methods in addition to other control strategies in order to successfully address this problem.

CHAPTER 4: Evaluation of various nematicides for their effect on the seed- and leaf-gall nematode, *Subanguina wevelli* in *Eragrostis curvula* seed.

1. Introduction

Nematicides are primarily used to limit damage to plants by reducing the number of plant-parasitic nematodes (PPN) invading plant parts such as roots, tubers, pods, leaves, seed and others. Nematicides further also contribute to reduce the risk of transmitting nematode-borne viruses to a host plant, for example earcockle disease which is transmitted by the SLGN *Anguina tritici* through the bacterium *Clavibacter tritici* (Riley & Reardon, 1995) and tobacco rattle tobravirus that is transmitted by the stubby-root nematode *Paratrichodorus minor* (Haydock *et al.*, 2006). The economic return on nematicide application is generally measured in terms of increase in crop yield and/or quality, resulting in a higher income for producers (Haydock *et al.*, 2006).

Nematicides are divided into two general groups, namely (i) fumigants that kill nematodes directly when they come into contact with them and (ii) non-fumigants (Whitehead, 2002; Schomaker & Been, 2006; Haydock *et al.*, 2006). Fumigants are volatile and produce a gas that kills PPN after application (Johnson, 1985). These gases diffuse through soil, dissolve in soil water and reach the bodies of PPN through the cuticle (Johnson, 1985). Non-fumigants are classified into either i) contact or ii) systemic nematicides and may constitute granular or liquid formulations (Whitehead, 2002; Schomaker & Been, 2006; Haydock *et al.*, 2006). Contact nematicides are applied to the soil either as a row, spot or broad-cast treatments, with the aim to put them into direct contact with PPN (Johnson, 1985). Systemic nematicides on the other hand are taken up by various plant parts, for example roots, leaves or pegs (Johnson, 1985; Nel *et al.*, 2007). The active substance of a systemic nematicide is subsequently translocated to the plant parts where it kills or prevents PPN from feeding (Johnson, 1985). Systemic nematicides are indigested by PPN during feeding or may also enter the nematode's body

through plant tissue or soil particles the latter comes into contact with (Johnson, 1985).

PPN are diverse in terms of feeding preference, habit and niche (Hooper & Evans, 1993). Therefore, the crop as well as the particular nematode group to be controlled affect the choice of a nematicide, its dosage rate as well as the application method to be used (Hooper & Evans, 1993). However, a wide variety of chemically based, broad-spectrum fumigant (F), systemic (S) as well as contact (C) nematicides is available locally, for example terbufos (Counter®; S), carbofuran (Curaterr®; S), ethylene dibromide (EDB®; F), ethoprophos (Mocap®; C), cadusafos (Rugby ®; C), aldicarb (Temik®; S) and others (Nel *et al.*, 2007). Nematicides produced from natural products (plant or animal material) are also available and are becoming more popular since they do not have a detrimental impact on the environment (Ferraz & De Freitas, 2004). Such "natural nematicides" include products such as i) avermectin, which is derived from the soil organism *Streptomyces avermitilis* (Morton *et al.*, 2006), ii) various neem-based products derived from seed, leaves and oil of the neem tree *Azadirachta indica* (Ferraz & De Freitas, 2004), iii) *Crotaliaria* spp., iv) *Tagetes* spp., v) *Mucuna* spp. and others (Ferraz & De Freitas, 2004).

Limited information is, however, available on the use and efficacy of nematicides specifically in suppressing SLGN belonging to the genus *Anguina/Subanguina* that infect various grass species.

2. Specific objective

To evaluate the efficacy of various chemical compounds in a microplot trial for their potential use in a management strategy for *S. wevelli* that infect seed of *E. curvula*.

3. Materials and methods

Microplots used during this study consisted of circular concrete tubes 1m in diameter and 1.25m high (Fig. 4.1). These tubes were partially sunken vertically into the soil. Each microplot was filled with soil and fumigated with

ethylene dibromide at a rate of 55kg/ha two weeks before nematode inoculation. The soil consisted of 2% clay, 2% silt, 96% sand and 0.48% organic material, with a pH of 6.55 (H_2O).

Figure 4.1. Layout of a microplot trial in which the efficacy of various nematicides were evaluated in the suppression of a *Subanguina wevelli* population infecting *Eragrostis curvula* (Ermelo variety) grass seed at Potchefstroom (North West Province) during February to June 2008.

The soil in each microplot was artificially inoculated with 20 *S. wevelli*-infected galls, nine days prior to planting. *Eragrostis curvula* (Ermelo variety) seed used as inoculum source was obtained from Mr. Tony Siebert[1]. These seeds (0.12g) were planted in the SLGN-infected microplots according to recommendations by Rethman (1988). The locally registered fumigant ethylene dibromide as well as granular formulations of the contact nematicides ethoprophos and cadusafos was broad-cast, applied and incorporated with a garden fork into the top 40-cm layer of soil seven days before planting. Other non-fumigant nematicides, namely aldicarb, carbofuran and terbufos were applied as granular formulations directly after planting (row

[1] Mr. Tony Siebert, Advance Seed, 26 College Avenue, Potchefstroom, 2531, South Africa.

treatments). Dosage rates used in this study and other information on these nematicides are provided in Table 4.1.

Table 4.1: Various nematicide treatments (Nel *et al.*, 2007) evaluated for their efficacy against the seed- and leaf-gall nematode *Subanguina wevelli* infecting *Eragrostis curvula* seed in a microplot trial during February to June 2008 at Potchefstroom (North West Province).

Treatment	Trade/registered name	Active substance	Formulation	Dosage rate	Chemical group
1	Untreated control				
2	Mocap GR	Ethoprophos @ 150 g/kg	Granular	35 kg/ha	Organophosphate
3	Temik GR	Aldicarb @ 150 g/kg	Granular	12 kg/ha (1 g/m row)	Oxime carbamate
4	Vydate SL	Oxamyl @ 310 g/l	Soluble concentrate	4 l/ha	Oxime carbamate
5	EDB	Ethylene dibromide @ 1 800 g/l	Liquid	55 l/ha	Halogenated hydrocarbon
6	Rugby 10 G	Cadusafos @ 100 g/kg	Granular	50 kg/ha	Organophosphate
7	Curaterr	Carbofuran @ 100 g/kg	Granular	20 kg/ha (1.5 g/m row)	Carbamate
8	Counter	Terbufos @ 150 g/kg	Granular	8.5 kg/ha (0.66 g/m row)	Organophosphate
9	Avicta	Avermectin	Emulsifiable concentrate	0.25 mg/seed	Macrocyclic lactone (Stretton *et al.*, 1987)

The seed-coating agent avermectin was applied to seed prior to planting at the rate recommended by Van Heerden[2]. Dosage rates of aldicarb, carbofuran and terbufos that were applied at planting during this study were equal to the registered rates for maize (Nel *et al.*, 2007) because it is also a monocotyledonous crop. Rates for ethoprophos and ethylene dibromide were

[2] Van Heerden, D. 2008. Manager Regulatory Affairs, Syngenta South Africa (Pty) Ltd, Private Bag X60, Halfway House, 1685, South Africa.

recommended by Fourie[3], while the rate for cadusafos was recommended by Jupp[4]. The post-planting, systemic, liquid-formulation nematicide oxamyl was applied seven days after seed germination, followed by a second application five weeks after planting and a third application nine weeks after planting. The dosage rate for oxamyl was obtained from Botes[5].

The trial layout was a randomised complete block design (RCBD) (Fig. 4.2) and included five replicates for each of the eight treatments and an untreated control (Table 4.1). The plants were irrigated by using drip irrigation (± 25mm) twice a week supplementary to rainfall. The inflorescences of all plants in each plot were harvested 30 weeks after planting, weighed and soaked for 48 hours in ± 50ml tap water to extract *S. wevelli*. Nematodes per pot were counted under a dissection microscope.

Nematode data were subjected to an analysis of variance (ANOVA) (Statgraphics Plus 5 for Windows). Means were separated by the LSD test ($P \leq 0.05$), while degrees of freedom (error) >18 (Van Ark, 1981) were always pursued.

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[3] Fourie, P. 2008. Portfolio Manager: Nematicides, Bayer CropScience, P.O. Box 143, Isando, 1600, South Africa.

[4] Jupp, D. 2007. Country manager: Disa Biotech, Private Bag X16, Constantia, South Africa.

[5] Botes, A. 2007. Technical representative, Du Pont, P.O. Box 3332, Halfway House, South Africa.

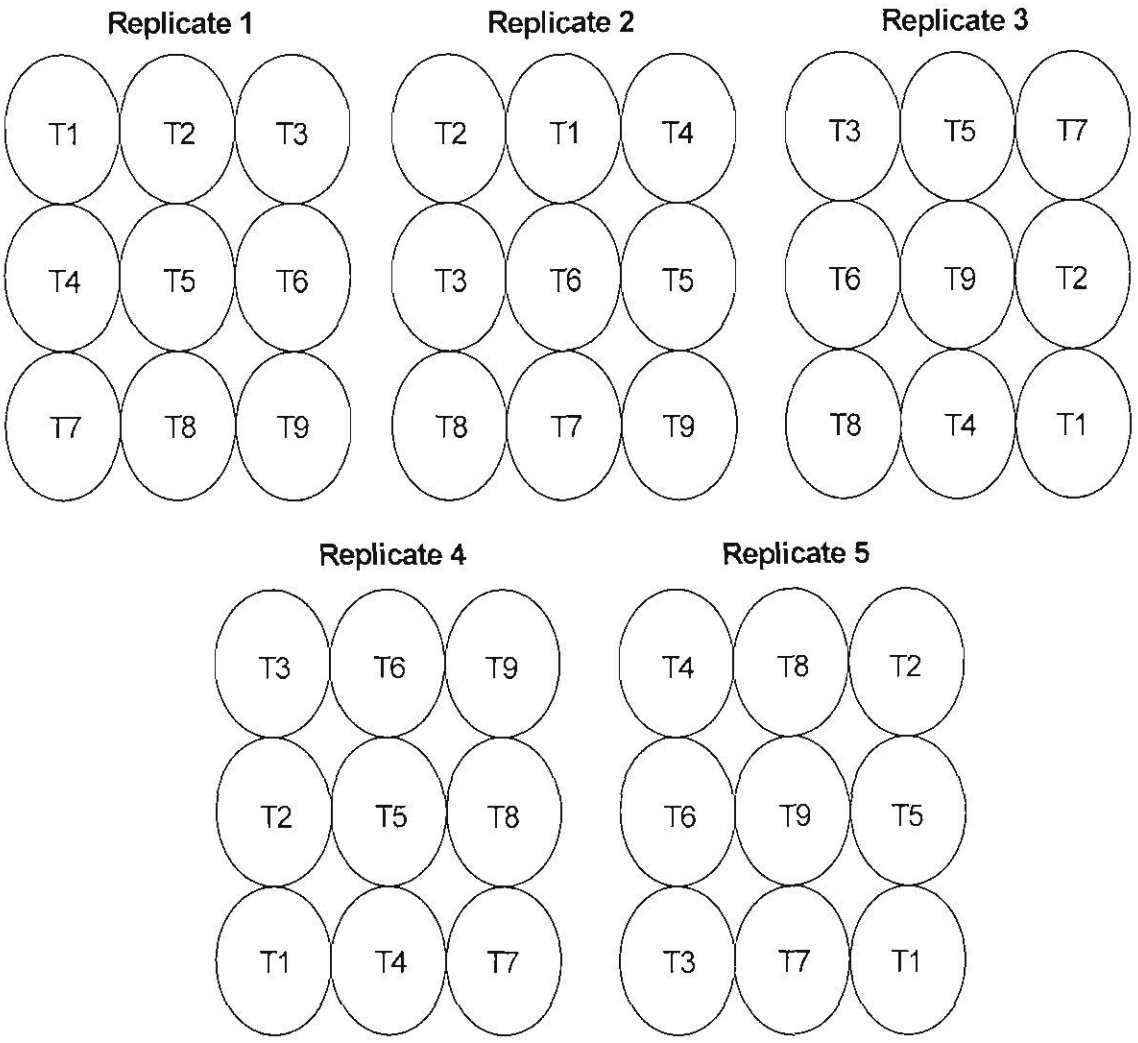

Figure 4.2. The trial layout, consisting of a randomised complete block design (RCBD) used during this study to evaluate the efficacy of various nematicides on *Subanguina wevelli* infection of *Eragrostis curvula* (Ermelo variety) grass in microplots during February to June 2008 at Potchefstroom (North West Province).

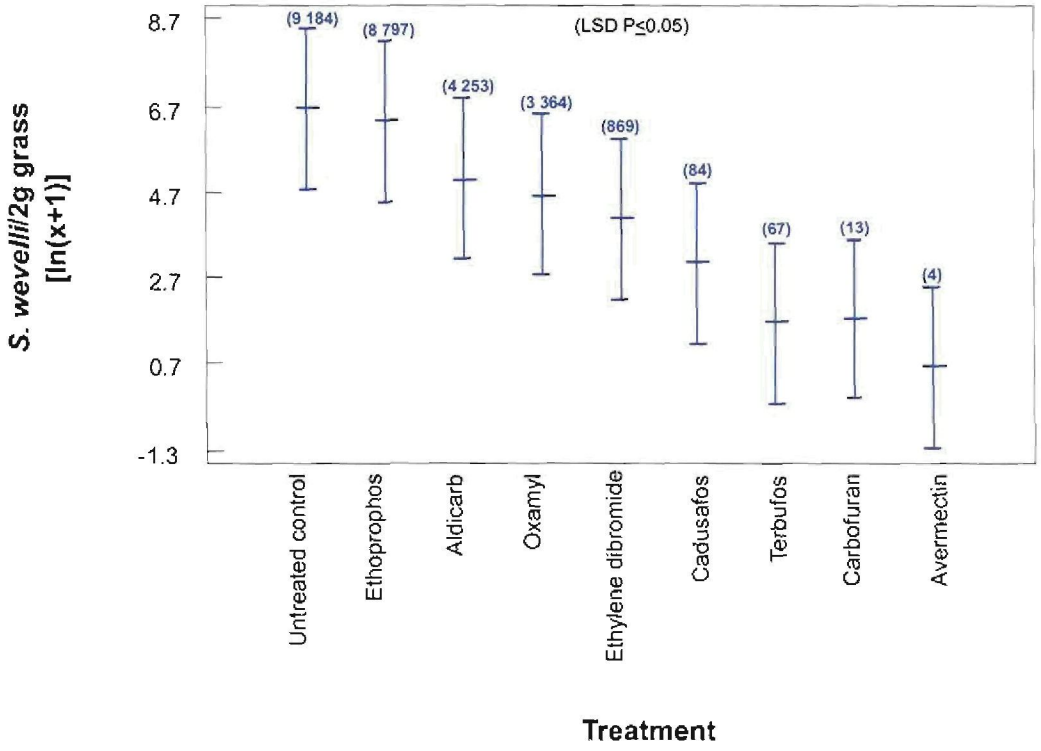

Figure 4.3. Number of *Subanguina wevelli* per 2g seed samples of *Eragrostis curvula* (variety Ermelo) seed after application of various nematicides in a microplot trial conducted in Potchefstroom (North West Province) during February to June 2008.

Table 4.2: F-ratios and P-values for the number of *Subanguina wevelli* juveniles for each of the pots.

Main effects	***S. wevellii*/2g grass [ln(x+1)]**	
	F-ratio	**P-value**
Treatment	2.69	0.0218
Replicate	2.39	0.0712

Substantial variation occurred in the efficacy of the respective nematicides in suppressing numbers of *S. wevelli* in *E. curvula* (Ermelo variety) grass seed (Fig. 4.3A; Table 4.2). The mean numbers of nematodes extracted from 2g seed of *E. curvula* for each of the eight nematicide treatments 30 weeks after planting ranged from 4 for avermectin to 8 797 for ethoprophos. The untreated control had a mean of 9 184 (Fig. 4.3A).

Avermectin differed significantly from the untreated control, ethoprophos, aldicarb and oxamyl, while terbufos and carbofuran differed significantly only from the untreated control. Ethoprophos, aldicarb, oxamyl, ethylene dibromide and cadusafos did not differ significantly from the untreated control. Ethylene dibromide, terbufos, carbofuran and avermectin did not differ significantly from each other. Aldicarb, oxamyl, EDB, cadusafos, terbufos and carbofuran also did not differ significantly from each other.

Seed mass of the various treatments ranged from 1g for oxamyl to 5.4g per pot for terbufos, while it was 2.0g per pot for the untreated control (data not shown). Since the P-value for replicates, however, differed significantly these data do not warrant further discussion.

5. Discussion

Although various nematicides have been evaluated for their efficacy against different SLGN genera or spp. (Malik *et al.*, 1997; Khan *et al.*, 1998), no reports specifically referring to *S. wevelli* could be found.

Results from this study showed that the contact nematicide avermectin, applied as a seed-coat treatment, as well as the systemic nematicides carbofuran and terbufos showed superior potential for reducing *S. wevelli* population levels sufficiently in *E. curvula* (Ermelo variety) grass. Avermectin was only recently registered for management of PPN as a seed dressing on cotton in the USA under the trade name Avicta Complete Pak (Anonymous, 2007a). As a seed dressing avermectin also proved to be effective in controlling *M. incognita* in vegetables (Monfort *et al.*, 2006). As a seed treatment the active substance comes into direct contact with J2 and J3 of *S.*

wevelli as soon as they emerge from the infected grass seed (Anonymous, 2007a). This may be one of the reasons why this nematicide was highly effective in this study. Furthermore, active substance avermectin moves from the treated seed along with the growing roots during germination, thus also protecting the young seedling from soil-dwelling PPN attacking from outside the root system (Anonymous, 2007a). The mode of action of avermectin is that it blocks transmission of electric stimuli between interneurons and excitatory motorneurons in the ventral chord of PPN (Spurr, 1984). Avermectin is a relative safe chemical to use in a nematode management system due to its low water solubility and rapid degradation, which makes it unlikely to cause contamination of soil water (Garbedian & Van Gundy, 1983).

The systemic carbamate carbofuran and the organophosphate terbufos respectively reduced *S. wevelli* numbers on par with avermectin during this study. Their active substances have most probably entered the body of SLGN while the latter were still in the seed galls and/or after the J2 left the seed to migrate upwards against the seedling. Absorption of these nematicides through the roots of germinating seed of *E. curvula* and translocation to the inflorescence where they killed or prevented the nematodes from feeding could have been another way these nematicides acted during this particular study. These two products are also known for their low water solubility, reducing the potential leaching of these products through the soil profile (Anonymous, 1994; Anonymous, 2007b).

The organophosphate, contact nematicide cadusafos also resulted in substantial suppression of *S. wevelli* numbers in this study. This type of nematicide disrupts i) chemoreception, ii) the ability of the nematode to find the host plant, iii) hatching and iv) movement of these parasites and is absorbed through the cuticle of the nematode (Haydock *et al.*, 2006).

No specific or clear explanation could be offered to explain why ethylene dibromide, oxamyl, aldicarb and ethoprophos applications did not perform well in terms of *S. wevelli* control in grass seed during this study. Most of them have similar modes of actions to carbofuran and terbufos (Haydock *et al.*,

2006). These products generally provided good control of PPN under optimal environmental conditions (Anonymous, 2008c; Anonymous, 2008d). There are, however inherent physical properties that differ, e.g. aldicarb is highly soluble in water and leach under conditions of excessive rainfall/irrigation.

The poor performance of soil fumigation with ethylene dibromide, which usually gives excellent control of PPN (Johnson, 1985; Haydock *et al.*, 2006) during this experiment could possibly be ascribed to the target SLGN having been sufficiently protected within the seed galls so that a large number of individuals escaped the effect of the active substance (Whithead, 2002). Although specific information on the effect of fumigants is not available for SLGN in grass seed, information on the effect of these nematicides on nematode eggs within similar structures have been published (Harrison, 1956; Hague, 1959; Whitehead 2002). Furthermore, most fumigants generally break down rapidly in soil, therefore not enough of the chemical is left after the waiting period to affect nematodes should they re-enter the treated areas (Rich *et al.*, 2004). Other potential constraints include soil factors, such as texture, structure, organic matter, moisture content and temperature during application (Haydock *et al.*, 2006), which can adversely influence effective fumigation. Freckman *et al.* (1980) for example discovered that significantly higher numbers of anhydrobiotic *Aphelenchus avenae* juveniles and adults survived fumigation compared to active nematodes.

The fact that seed mass data showed significant differences for the five replicates used in this study will not be further discussed. No physical symptoms such as yellowing, stunting, etc. were observed during this study, so phytotoxicity does not seem to be a factor at the rates and application methods used in this study.

6. Conclusions

Although the trial was not repeated nor done under field conditions the results of this study strongly suggest that avermectin, carbofuran and terbufos are able to provide superior control of *S. wevelli* in *E. curvula* seed. However, since the efficacy of nematicides could be affected by a wide range of factors such as soil and climate conditions, it is essential that more similar tests should be done, preferably at more field localities to confirm these results. It is important, though, that strong indications were obtained that chemical control of *S. wevelli* in grass seed is a viable option for the industry in terms of nematode population suppression. The economies there-of and the desirability of chemical control as a component of integrated control of SLGN need investigation.

CHAPTER 5: Conclusions

The presence of seed- and leaf-gall nematodes (SLGN) in *E. curvula* seed samples from 12 of the 13 localities sampled during this study, confirms earlier reports that these parasites pose a real risk for local grass seed producers and related industries. The adverse impact on Eragrostis seed export that is currently experienced due to the presence of SLGN has a negative effect on the economy.

The occurrence (100%) of *S. wevelli* at all localities sampled as well as their high population densities and PV support and confirm the threat these parasites pose for relevant producers and the industry in current and potential Eragrostis production areas of South Africa. Results of molecular methods used showed that these parasites could be considered as *S. wevelli*. The use of molecular techniques, constituting PCR followed by DNA sequencing, confirmed the identity of these parasites as *S. wevelli*. Phylogenetic tree data indicated that these 12 SLGN populations had bootstrap values of less than 90% and did not differ significantly from each other or from the *S. wevelli* reference population (USA). DNA sequence data for the 12 local *S. wevelli* populations was furthermore in agreement with those of Subbotin *et al.* (2004). Low similarity values obtained between the ITS regions of these populations and the reference population substantiated their identification further. *Subanguina wevelli* populations from all the localities are being reared *in vivo* on *E. curvula* (Ermelo variety) in order to obtain mature specimens for confirmation of molecular identification.

The use of commercialisable techniques used during this study to reduce population levels of *S. wevelli* in Eragrostis seed proved to be unsuccessful. In terms of sieving, all three sieves used contained significantly less *S. wevelli* than the unsieved control treatment. Although the seed contained on the 250-µm-mesh contained the lowest *S. wevelli* numbers (13% infested), followed by the 500-µm-mesh sieve (28% infested) and the 600-µm-mesh sieve (31% infested), the mere presence of SLGN is unacceptable. The flotation-separation where a range of NaCl solutions were used to mechanically separate SLGN-infected from SLGN-free seed resulted in treatments not

differing significantly from the untreated control (tap water only) in terms of *S. wevelli* numbers. *Subanguina wevelli* numbers in floating seed ranged from 702 to 1 952 for the 7.5% and 20% NaCl treatments, respectively, and from 84 to 392 for the 12.5% and 5% treatments in sunken seed, respectively. The presence of such high SLGN numbers in the sunken seed therefore also renders this method ineffective according to our investigations. When sieving and the NaCl techniques were combined, high numbers of *S. wevelli* were still present in seed batches subjected to both the sieve treatments followed by the 15% NaCl solution treatment. Planting of such seed will therefore still lead to *S. wevelli* infection in Eragrostis grass.

The application of a range of nematicides for evaluation of their efficacy in reducing *S. wevelli* numbers were promising according to a preliminary trial that has been conducted in micro plots. Treatment of *E. curvula* seed with 0.25mg abamectin per seed, carbofuran (1.5g/m) and terbufos (0.66g/m) resulted in the greatest reduction of *S. wevelli* numbers per 2g seed and these differed significantly from the untreated control. Aldicarb, cadusafos, ethylene dibromide, ethoprophos and oxamyl treatments still had relatively high numbers of *S. wevelli* and did not differ significantly from the untreated control. These nematicides should, however, be evaluated at more than one field locality to address the influence of various soil and climatic factors on its efficacy in reducing SLGN numbers.

Results from this study indicate that a single control strategy is not adequate to ensure that grass-seed consignments are free of SLGN. More than one control strategy, included as components of an integrated pest management system has to be applied to address this nematode problem. Only then will the impact this parasite has on the local grass industry be reduced.

CHAPTER 6: References

AGRIOS, G.N. 1997. Plant Pathology. 4th ed. San Diego: Academic Press. 635 p.

AL-TALIB, N.Y., AL-TAEE, A.K.M., NEINER, S.M., STEPHAN, Z.A. & AL-BALDAWI, A.S. 1986. New record of *Anguina tritici* on barley from Iraq. *Int. Nem. Netw. Newsl.*, 3:25-27.

ANONYMOUS. 2007a. Planting corn on cotton acres may open a whole new can of worms. *Corn South*, 13-16.

ANONYMOUS. 2007b. Carbofuran 100 GR. http://www.villacrop.co.za/products/docs/Carbofuran%20100%20GR_UCP_MSDS.pdf Date of access: 2 December. 2008.

ANONYMOUS. 2008a. South African National Seed Organisation (SANSOR): South African variety list as maintained by the Registrar of Plant Improvement. http://www.sansor.org/pdf/VarietyListApril2008.pdf Date of access: 30 October. 2008.

ANONYMOUS. 2008b. South African National Seed Organisation (SANSOR): Annual report 2007/08. http://www.sansor.org/annualreport/2007_08.pdf Date of access: 11 November. 2008.

ANONYMOUS. 2008c. Bayer CropScience: Temik. http://www.bayercropscience.co.za/products Date of access: 2 December. 2008.

ANONYMOUS. 2008d. Extension toxicology network: Oxamyl. http://pmep.cce.cornell.edu/profiles/extoxnet/metiram-propoxur/oxamyl-ext.html Date of access: 2 December. 2008.

BARRETT, J. 1991. Anhydrobiotic nematodes. (*In* Evans, K., *ed.* Agricultural Zoology reviews: Volume 4. Andover: Intercept. p. 161-176.)

BRIDGE, J. 1996. Nematode management in sustainable and subsistence agriculture. *Annual review of Phytopathology*, 34:201-225.

BROMILOW, C. 2001. Problem plants of South Africa. Pretoria: Briza Publications. 258 p.

BROWN, R.H. & KERRY, B.R. 1987. Principles and practices of nematode control in crops. New York: Academic Press. 447 p.

CHEN, Z.X. & DICKSON, D.W. 1998. Review of *Pasteuria penetrans*: biology, ecology and biological control potential. *Journal of Nematology*, 30:313-340.

CHEN, Z.X. & DICKSON, D.W. 2004. Biological control of Nematodes by fungal antagonists. (*In* Chen, Z.Y., Chen, S.Y. & Dickson, D.W., *eds.* Nematology advances and perspectives, Volume 2: Nematode management and utilization. Wallingford: CABI Publishing. p. 979-1040.)

CHEN, Z.X., CHEN, S.Y. & DICKSON, D.W. 2004., *eds.* Nematology advances and perspectives, Volume 1: Nematode morphology, physiology and ecology. Wallingford: CABI Publishing. 636 p.

CHIZHOV, V.N. & SUBBOTIN, S.A. 1990. Phytoparasitic nematodes of the subfamily Anguininae (Nematoda: Tylenchida). Morphology, trophic specialization, systematics. *Zoologeschki Zhurnal*, 29:21-29.

COOK, B.G., PENGELLY, B.C., BROWN, S.D., DONNELLY, J.L., EAGLES, D.A., FRANCO, M.A., HANSON, J., MULLEN, B.F., PARTRIDGE, I.J., PETERS, M. & SCHULTZE-KRAFT, R. 2005. Tropical Forages: an interactive selection too. http://www.tropicalforages.info Date of access: 27 May. 2008.

COOK, R. & STARR, J.L. 2006. Resistant cultivars. (*In* Perry, R.N. & Moens, M., *eds*. Plant Nematology. Wallingford: CAB International. p. 370-389.)

COURTNEY, W.D. & HOWELL, H.B. 1952. Investigations on the bent grass nematode, *Anguina agrostis* (Steinbuch 1799) Filipjev 1936. *Plant Disease Reporter*, 36(3):75-83.

COURTNEY, W.D., PEABODY, D.V. & AUSTENSON, H.M. 1962. Effect of herbicides on nematodes in bent grass. *Plant Disease Reporter*, 46:256-257.

DAHL, B.E. & COTTER, P.F. 1984. Management of weeping lovegrass in west Texas: Management Note 5. Lubbock: Texas Tech Press. 12 p.

DECRAEMER, W. & HUNT, D.J. 2006. Structure and Classification. (*In* Perry, R.N. & Moens, M., *eds*. Plant Nematology. Wallingford: CAB International. p. 3-32.)

DE GRISSE, A.T. 1969. Redescription ou modification de quelques techniques utilisées dans l'étude des nematodes phytoparasitaires. Meded. Rijksfac. LandbWet. *Gent*, 34:351-359.

DEMEURE, Y. & FRECKMAN, D.W. 1981. Recent advances in the study of anhydrobiotic nematodes. (*In* Zuckerman, B.M. & Rohde, R.A., *eds*. Plant-parasitic Nematodes. Volume III. New York: Academic Press. p. 205-226.)

DOWLER, W.M. & VAN GUNDY, S.D. 1984. Importance of agricultural plant nematology. (*In* Nickle, W.R., *ed*. Plant and insect nematodes. New York: Marcel Dekker. p. 1-12.)

DREWES, R.H. 1985?. Die diereproduksiepotensiaal van Oulandsgras, Kikoejoe, Lusern en Smutsvingergras in die Oos-Vrystaat. Departement Landbou en Watervoorsiening, Potchefstroom.

DUNCAN, L.W. & MOENS, M. 2006. Migratory endoparasitic nematodes. (*In* Perry, R.N. & Moens, M., *eds*. Plant Nematology. Wallingford: CAB International. p. 123-152.)

EBSARI, B.A. 1991. Catalogue of the order Tylenchida (Nematoda). Canada: *Research Branch, Agriculture Canada*. 196 p.

EVANS, K., TRUDGILL, D.L. & WEBSTER, J.M. 1993. Plant-parasitic nematodes in temperate agriculture. UK: CAB International. 659 p.

FATTAH, F.A. 1988. Effects of inoculation methods on the incidence of ear-cockle and 'tundu' on wheat under field conditions. *Plant and Soil*, 109:195-198.

FERRAS, S. & DE FREITAS, L.G. 2004. Use of antagonistic plants and natural products. (*In* Chen, Z.Y., Chen, S.Y. & Dickson, D.W., *eds*. Nematology advances and perspectives, Volume 2: Nematode management and utilization. Wallingford: CABI Publishing. p. 931-977.)

FERRIS, H. 1984. Nematode Damage Functions: The Problems of Experimental and Sampling Error. *Journal of Nematology*, 16(1):1-9.

FERRIS, H. 1999. NEMAPLEX: The "Nematode-plant expert information system: *Anguina agrostis*.
http://plpnemweb.ucdavis.edu/Nemaplex/Taxadata/G006S1.htm
Date of access: 29 July. 2008.

FOURIE, H. & MC DONALD, A.H. 2002. Evaluation of management strategies for the seed- and leaf-gall nematode on *Eragrostis* spp. in South Africa. Project proposal to the Department of Agriculture. 18p.

FOURIE, H., ZIJLSTRA, C. & MC DONALD, A.H. 1998. ITS-PCR sequence-based identification of *Meloidogyne chitwoodi* from Mooi River, South Africa, and screening of crops for host suitability. *African Plant Protection*, 4:107-111.

FRECKMAN, D.W., DEMEURE, Y. MUNNECKE, D. & VAN GUNDY, S.D. 1980. Resistance of anhydrobiotic *Aphelenchus avenae* to methyl bromide fumigation. *Journal of Nematology*, 12:19-22.

GARABEDIAN, S. & VAN GUNDY, S.D. 1983. Use of avermectins for the control of *Meloidogyne incognita* on tomatoes. *Journal of Nematology*, 15:503-510.

GERAERT, E. 2006. Functional and detailed morphology of the Tylenchida (Nematoda). (*In* Hunt, D.J. & Perry, R.N., *eds*. Nematology monographs and perspectives, Volume 4. Leiden: Brill Academic Publishers. 215 p.)

GOTKE, N. & SWARUP, G. 1989. Effect of neem (*Azadirachta indica*) seed kernel powder treatment on penetration and development of *Anguina tritici* on wheat. *Indian Journal of Nematology*, 14:95-96.

GRIFFIN, G.D. 1984. Nematode parasites of Alfalfa, Cereals, and grasses. (*In* Nickle, W.R., *ed.* Plant and insect nematodes. New York: Marcel Dekker. p. 243-307.)

HALBRENDT, J.M. & LAMONDIA, J.A. 2004. Crop rotation and other cultural practices. (*In* Chen, Z.Y., Chen, S.Y. & Dickson, D.W., *eds*. Nematology advances and perspectives, Volume 2: Nematode management and utilization. Wallingford: CABI Publishing. p. 637-716.)

HAGUE, N.G.M. 1963. Fumigation of agricultural products. XVIII: Effect of methyl bromide on the bent grass nematode, *Anguina agrostis* (Steinbuch 1799) Filipjev 1936, and on the germination of bent grass, *Agrostis tenuis*. *J. Sci. Fd Agric*, 14(8):577-579.

HAYDOCK, P.P.J., WOODS, S.R., GROVE, I.G. & HARE, M.C. 2006. Chemical control of nematodes. (*In* Perry, R.N. & Moens, M., *eds*. Plant Nematology. Wallingford: CAB International. p. 392-408.)

HEYNS, J. 1971. A guide to the plant and soil nematodes of South Africa, digital reproduction. Cape Town: A.A. Balkema. 233 p.

HOCKLAND, S., INSERRA, R.N., MILLAR, L. & LEHMAN, P.S. 2006. International plant health – Putting legislation into practice. (*In* Perry, R.N. & Moens, M., *eds*. Plant Nematology. Wallingford: CAB International. p. 328-434.)

HOOPER, D.J. & EVANS, K. 1993. Extraction, identification and control. (*In* Evans, K., Trudgill, D.L. and Webster, J.M., *eds*. Plant-parasitic nematodes in temperate agriculture. Wallingford: CAB International. p. 47-59.)

HOOPER, D.J., HALLMANN, J. & SUBBOTIN, S. 2005. Methods for extraction, processing and detection of plant and soil nematodes. (*In* Luc, M., Sikora, R.A. & Bridge, J., *eds*. Plant-parasitic Nematodes in Subtropical and Tropical Agriculture. 2nd ed. Wallingford: CAB International. p. 53-86.)

HUNT, D.J., LUC, M. & MANZANILLA-LÓPEZ, R.H. 2005. Identification, morphology and biology of plant-parasitic nematodes. (*In* Luc, M., Sikora, R.A. & Bridge, J., *eds*. Plant-parasitic Nematodes in Subtropical and Tropical Agriculture. 2nd ed. Wallingford: CAB International. p. 11-52.)

INSERRA, R., BRITO, J.A., DONG, K., HANDOO, Z.A., LEHMAN, P.S., POWERS, T. & MILLAR, L. 2003. List of exotic nematode plant pests of agricultural and environmental significance to the United States. http://nematode.unl.edu/pest33.htm Date of access: 8 Jan. 2007.

JENKINS, W.R. & TAYLOR, D.P. 1967. Plant Nematology. New York: Reinhold Publishing Corporation. 270 p.

JENSEN, H.J. 1961. Nematode affecting Oregon agriculture. Ore. Agric. Exp. Sta. Bull. 579 p.

JOHNSON, A.W. 1985. The role of nematicides in nematode management. (*In* Sasser, J.N. & Carter, C.C., *eds.* An advanced treatise on *Meloidogyne*, Volume 1: Biology and Control. Raleigh: North Carolina State University Graphics. p. 249-267.)

KEETCH, D.P. & BUCKLEY, N.H. 1984. A checklist of the plant-parasitic nematodes of southern Africa. Pretoria: Government Printer. 213p.

KEETCH, D.P. & HEYNS, J. 1982. Nematology in southern Africa. Pretoria: Government Printer. 170 p.

KHAN, M.R., ATHAR, M. & KHAN, B. 1998. Effect of foliar application of monocrotophos on the ear-cockle disease on wheat. *Nematol medit.*, 26:173-177.

KLEYNHANS, K.P.N., VAN DEN BERG, E., SWART, A., MARAIS, M. & BUCKLEY, N.H. 1996. Plant nematodes in South Africa. Pretoria: Business Print. 164 p.

KRALL, E.L. 1991. Wheat and grass nematodes: *Anguina, Subanguina*, and related genera. (*In* Nickle, W.R., *ed.* Manual of Agricultural Nematology. New York: Marcel Dekker. p. 721-760.)

LUC, M. BRIDGE, J. & SIKORA, R.A. 2005. Reflections on Nematology in Subtropical and Tropical agriculture. (*In* Luc, M., Sikora, R.A. & Bridge, J., *eds.* Plant parasitic nematodes in subtropical and tropical agriculture. 2nd ed. Wallingford: CAB International. p. 1-10.)

LUC, M., HUNT, D.J. & MACHON, J.E. 1990. Morphology, Anatomy and Biology of Plant-parasitic Nematodes – a Synopsis. (*In* Luc, M., Sikora, R.A. and Bridge, J., *eds.* Plant-parasitic Nematodes in Subtropical and Tropical Agriculture. 2nd ed. Wallingford: CAB International. p. 1-13.)

MAGGENTI, A. 1981. General Nematology. New York: Springer-Verslag. 372 p.

MALIK, M.S., MALIK, O.P. & SANGWAN, N.K. 1997. Toxicity of ß-ketoesters and ß-dicarbonyl compounds to juveniles of the seed-gall nematode *Anguina tritici*. *Nematologica*, 43:193-198.

MANZANILLA-LÓPEZ, R.H., EVANS, K. & BRIDGE, J. 2004. Plant Diseases Caused by Nematodes. (*In* Chen, Z.Y., Chen, S.Y. & Dickson, D.W., *eds.* Nematology advances and perspectives, Volume 2: Nematode management and utilization. Wallingford: CABI Publishing. p. 637-716.)

MC DONALD, A.H. & NICOL, J.M. 2005. Nematode parasites of cereals. (*In* Luc, M., Sikora, R.A. & Bridge, J., *eds.* Plant-parasitic Nematodes in Subtropical and Tropical Agriculture. 2nd ed. Wallingford: CAB International. p. 131-191.)

MCKAY, A.C. 1993. Livestock deaths associated with *Clavibacter/Anguina* sp. infection in seedheads of *Agrostis avenacea* and *Polypogon monspeliensis*. *Plant Disease*, 77(6):635-641.

MONFORT, W.S., KIRKPATRICK, T.L., LONG, D.L. & RIDEOUT, S. 2006. Efficacy of a noval nematicidal seed treatment against *Meloidogyne incognita* on cotton. *Journal of Nematology*, 38:245-249.

MYNHARDT, J.E., VAN ROOYEN, M.W. & THERON, G.K. 1994. Competitive ability of two grass species: *Anthephora pubescens* and *Eragrostis curvula*: 1. Yield and biomass allocations. *South African Journal of Botany*, 60:261-268.

NEL, A., KRAUSE, M. & KHELAWANLALL, N. 2007. A guide for the control of plant pests. 40th Edition. Department of Agriculture, Republic of South Africa. Pretoria: Government Printer. 275p.

NOLING, J.W. 2003. Nematodes and their management. http://edu.ifas.ufl.edu/CV112p Date of access: 8 Jan. 2007.

NORTON, D.C. 1978. Ecology of plant-parasitic nematodes. New York: John Wiley & Sons. p. 59-79.

PARUTHI, I.J. & BHATTI, D.S. 1992. Efficacy of different practises for the control of ear-cockle in wheat caused by *Anguina tritici*. *Current Nematology*, 3:1-4.

PINKERTON, J. N. & ALDERMAN, S. C. 1994. Epidemiology of *Anguina agrostis* on Highland Colonial Bentgrass. *Journal of Nematology*, 26(3):315-323.

POWERS, T.O., SZALANSKI, A.L., MULLIN, P.G., HARRIS, T.S., BERTOZZI, T. & GRIESBACH, J.A. 2001. Identification of seed gall nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. *Journal of Nematology*, 33(4):191-194.

RETHMAN, N.F.G. 1988a. The influence of management practices on seed production of *Eragrostis curvula* cv. Ermelo. *South African Forage Seed Assoc. Workshop. Roodeplaat.*

RETHMAN, N.F.G. 1988b. *Eragrostis curvula*. *Farming in South Africa*, leaflet no. 119

RICH, J.R., DUNN, R.A. & NOLING, J.W. 2004. Nematicides: Past and present uses. (*In* Chen, Z.Y., Chen, S.Y. & Dickson, D.W., *eds*. Nematology advances and perspectives, Volume 2: Nematode management and utilization. Wallingford: CABI Publishing. p. 1179-1200.)

RILEY, I.T. & MCKAY, A.C. 1990. Specificity of the adhesion of some plant pathogenic micro-organisms to the cuticle of nematodes in the genus *Anguina* (Nematoda: Anguinidae). *Nematologica*, 36:90-103.

RILEY, I.T. & REARDON, T.B. 1995. Isolation and characteristics of *Clavibacter tritici* associated with *Anguina tritici* in wheat from Western Australia. *Plant Pathology*, 44:805-810.

RILEY, I.T., REARDON, T.B. & MCKAY, A.C. 1988. Electrophoretic resolution of species boundaries in seed-gall nematodes, *Anguina* spp. (Nematoda: Anguinidae), from some Graminacceous hosts in Australia and New Zealand. *Nematologica*, 34:401-411.

ROBERTS, P.A. 1994. Integration and protection of novel management strategies. *Fundamental and Applied Nematology,* 17(3): 203-210.

SAEED, M. & ROESSNER, J. 1984. Anhydrobiosis in five species of plant associated nematodes. *Journal of Nematology*, 16(2):119-124.

SALEH, H.M. & FATTAH, F.A. 1989. Reaction of wheat genotypes to infection by *Anguina tritici*. *Revue Nématol.* 12(4):429-431.

SANDERSON, M.A., VOIGT, P. & JONES, R.M. 1999. Yield and quality of warm-season grasses in central Texas. *Journal of Range Management*, 52:145-150.

SCHEEPERS, L. 2005. Diereproduksie vanaf aangeplante weidings in die sentrale somersaaigebied: 'n Praktiese handleiding vir droëland en besproeing. Maanhaarrand: Kejafa Knowledge Works. p. 92-99.

SCHOMAKER, C.H. & BEEN, T.H. 2006. Plant growth and population dynamics. (*In* Perry, R.N. and Moens, M., *eds.* Plant nematology. Wallingford: CAB International. p. 275-301.)

SIDDIQI, M.R. 1985. Tylenchida: Parasites of plants and insects. Slough: Commonwealth Agricultural Bureaux. 645 p.

SIDDIQI, M.R. 2000. Tylenchida: Parasites of plants and insects. 2nd ed. Oxen: CABI Publishing. 848 p.

SIKORA, R.A., BRIDGE, J. & STARR, J.L. 2005. Management practice: An overview of integrated nematode management technologies. (*In* Luc, M., Sikora, R.A. & Bridge, J., *eds.* Plant-parasitic Nematodes in Subtropical and Tropical Agriculture. 2nd ed. Wallingford: CAB International. p. 793-825.)

SKERMAN, P.J., RIVEROS, F. & HOARE, D.B. 2008. *Eragrostis curvula* (Schrad.) Nees. http://www.fao.org/ag/AGP/AGPC/dog/GBASE/Safricadata/eragcur.htm Date of access: 22 May. 2008.

SPURR, H.W. 1985. Mode of action of nematicides. (*In* Sasser, J.N. & Carter, C.C., *eds.* An advanced treatise on *Meloidogyne*, Volume 1: Biology and Control. Raleigh: North Carolina State University Graphics. p. 269-276.)

STANTON, J.M., MCNICOL, C.D. & STEELE, V. 1998. Non-manual lysis of second-stage *Meloidogyne* juveniles for identification of pure and mixed samples based on the polymerase chain reaction. *Australian Plant Pathology,* 27:112-115.

STARR, J.L., BRIDJE, J. & COOK, R. 2002. Resistance to plant-parasitic nematodes: History, current use and future potential. (*In* Starr, J.L., Cook, R. & Bridge., *eds.* Plant resistance to parasitic nematodes. Wallingford: CAB International. p. 1-22.)

STRETTON, A.O.W., CAMPBELL, W.C. & BABU, J.R. 1987. Biological activity and mode of action of avermectins. (*In* Veech, J.A. & Dickson, D.W., *eds.* Vistas on Nematology: A commemoration of the twenty-fifth anniversary of the Society of Nematologists. Florida: E.O. Painter Printing Co. p. 136-146.)

SUBBOTIN, S.A., KRALL, E.L., RILEY, I.T., CHIZHOV, V.N., STAELENS, A., DE LOOSE, M. & MOENS, M. 2004. Evolution of the gall-forming plant-parasitic nematodes (Tylenchida: Anguinidae) and their relationships with hosts as inferred from Internal Transcribed Spacer sequences of nuclear ribosomal DNA. *Molecular Phylogenetics and Evolution,* 30:226-235.

SUBBOTIN, S.A. & MOENS, M. 2006. Molecular taxonomy and phylogeny. (*In* Perry, R.N. & Moens, M., *eds.* Plant Nematology. Wallingford: CAB International. p. 34-58.)

SUBBOTIN, S.A., WAYENBERGE, L. & MOENS, M. 2000. Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLP. *Nematology*, 2(2): 153-164.

SWART, A., SUBBOTIN, S.A., TIEDT, L.R. & RILEY, I.T. 2004. *Anguina woodi* sp. n. (Tylenchida: Anguinidae) from dune grass, *Ehrharta villosa*, in South Africa. *Nematology*, 6(1):129-144.

SWARUP, G. & SOSA-MOSS, C. 1993. Nematode parasites of cereals. (*In* Luc, M., Sikora, R.A. & Bridge, J., *eds.* Plant-parasitic nematodes in subtropical and tropical agriculture. 2nd ed. Wallingford: CAB International. p. 109-136.)

THOMPSON, J.D., GIBSON, T.J., PLEWNIAK, F., JENAMOUGIN, F., HIGGINS, D.G. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25:4876-4882.

TORELL, L.A., KIRKSEY, R.E., DONART, G.B. & LIBBIN, J.D. 2000. Economics of grazing weeping lovegrass with stockers in the Southern Great Plains. *Journal of Range Management*, 53:268-276.

VAN DEN BERG, E. 1985. Notes on the genus *Afrina* Brzeski, 1981 (Anguinidae: Nematoda) with descriptions of new and known species. *Phytophylactica,* 17:67-79.

VAN HEERDEN, M. 2002. Die aanwending en bestuur van oulandsgras (*Eragrostis curvula*). *SA KOÖP*: 14-17.

VAN OUDTSHOORN, F. 1999. Gids tot grasse van Suider Afrika. Pretoria: Briza Publikasies. 288 p.

VENTER, C., DE WAELE, D. & MEYER, A.J. 1992. Minimizing damage by *Ditylenchus destructor* to peanut seed with early harvest. *Journal of Nematology,* 24:528-532.

VIAENE, N., COYNE, D.L. & KERRY, B.R. 2006. Biological and cultural management. (*In* Perry, R.N. and Moens, M., *eds*. Plant nematology. Wallingford: CAB International. p. 346-359.)

VOIGT, P.W. & SHARP, W.C. 1994. Grasses of the plains and southwest. (*In* Barnes, R.F., Miller, D.A. & Nelson, C.J., *eds*. Forages: an introduction to grassland agriculture. Vol 1. Ames: Iowa State University Press. p. 395-408.)

WHITEHEAD, A.G. 2002. Plant nematode control. Wallingford: CABI International. 384 p.

WIESEMILLER, B. & ROTHE, H. 2006. Interpretation of bootstrap values in phylogenetic analysis. *Anthropologischer Anzeiger*, 64(2):161-165.

WILMOTT, S., GOOCH, P.S., SIDDIQI, M.R. & FRANKLIN, M., *eds*. 1972. C.H.I. Descriptions of plant-parasitic nematodes. Set 1, nos. 1-15. London: William Clowes & Sons Ltd.

WILMOTT, S., GOOCH, P.S., SIDDIQI, M.R. & FRANKLIN, M., *eds*. 1973. C.H.I. Descriptions of plant-parasitic nematodes. Set 2, Nos. 16-30. London: William Clowes & Sons Ltd.

WOMERSLEY, C., THOMPSON, S.N. & SMITH, L. 1982. Anhydrobiosis in Nematodes II: Carbohydrate and lipid analysis in undesiccated and desiccate nematodes. *Journal of Nematology*, 14(2):145-153.

WRIGHT, D.J. & PERRY, R.N. 2006. Reproduction, physiology and biochemistry. (*In* Perry, R.N. & Moens, M., *eds*. Plant Nematology. Wallingford: CAB International. p. 392-408.)

ZENG, J., SUBBOTIN, S.A., WAEYENBERGE, L., MOENS, M. 2000. Molecular characterization of Chinese *Heterodera glycines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions. *Russian Journal of Nematology*, 8:109-113.

ZIJLSTRA, C., DONKERS-VENNE, D.T.H.M. & FARGETTE, M. 2000. Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR. *Nematology*, 2:847-853.

APPENDIX A

1. Sequencing of SLGN DNA

Table 1: Percentage similarities between the SLGN populations from 12 localities as well as *Subanguina wevelli* (GCI-reference) and *Anguina agrostis* (GenBank- and Subbotin-reference).

	TW81 A2	TW81 A5	TW 81 A7	TW81 A3	TW81 O1	TW81 A8	TW81 B	TW81 A6	TW81 Sw(R)	TW81 A1	TW81 A9	TW81 P	AB28 A1	AB28 A9
TW81_Amersfoort2 (A2)	100	99.8	100	100	99.8	99.4	99.7	99.1	99.8	99.5	99.5	99.5	99.4	98.9
TW81_Amersfoort5 (A5)	99.8	100	99.8	99.8	99.7	99.2	99.5	98.9	99.7	99.4	99.4	99.4	99.2	98.7
TW81_Amersfoort7 (A7)	100	99.8	100	100	99.8	99.2	99.7	99.1	99.8	99.5	99.5	99.5	99.4	98.9
TW81_Amersfoort3 (A3)	100	99.8	100	100	100	99.8	99.8	99.8	100	99.8	99.8	99.8	99.7	99.2
TW81_Oordrag1 (O1)	99.8	99.7	99.8	100	100	100	99.7	100	99.8	99.7	99.7	99.7	99.5	99
TW81_Amersfoort8 (A8)	99.4	99.2	99.2	99.8	100	100	99.4	99.5	99.4	99.2	99.2	99.2	99	98.5
TW81_Bethal (B)	99.7	99.5	99.7	99.8	99.7	99.4	100	98.8	99.8	99.8	99.8	99.8	99.7	99.2
TW81_Amersfoort6 (A6)	99.1	98.9	99.1	99.8	100	99.5	98.8	100	98.9	98.4	98.6	98.4	98.2	97.7
TW81_Sw (R)[1]	99.8	99.7	99.8	100	99.8	99.4	99.8	98.9	100	99.7	99.7	99.7	99.5	99
TW81_Amersfoort1 (A1)	99.5	99.4	99.5	99.8	99.7	99.2	99.8	98.4	99.7	100	100	100	99.8	99.4
TW81_Amersfoort9 (A9)	99.5	99.4	99.5	99.8	99.7	99.2	99.8	98.6	99.7	100	100	100	99.8	99.4
TW81_Potchefstroom (P)	99.5	99.4	99.5	99.8	99.7	99.2	99.8	98.4	99.7	100	100	100	99.8	99.4
AB28_Amersfoort1 (A1)	99.4	99.2	99.4	99.7	99.5	99	99.7	98.2	99.5	99.8	99.8	99.8	100	100
AB28_Amersfoort9 (A9)	98.9	98.7	98.9	99.2	99	98.5	99.2	97.7	99	99.4	99.4	99.4	100	100
AB28_Amersfoort4 (A4)	99.2	99	99.2	99.5	99.4	98.9	99.5	98.1	99.4	99.7	99.7	99.7	100	100
AB28_Potchefstroom (P)	99.4	99.2	99.4	99.7	99.5	99	99.7	98.2	99.5	99.8	99.8	99.8	100	100
AB28_Amersfoort8 (A8)	99.4	99.2	99.4	99.7	99.5	99	99.7	98.2	99.5	99.8	99.8	99.8	100	100
AB28_Sw (R)[2]	99.5	99.4	99.5	99.8	99.7	99.2	99.8	98.4	99.7	100	100	100	100	100
AB28_Amersfoort3 (A3)	99.5	99.4	99.5	99.8	99.7	99.2	99.8	98.4	99.7	100	100	100	100	100
AB28_Amersfoort7 (A7)	99.4	99.2	99.4	99.7	99.5	99	99.7	98.2	99.5	99.8	99.8	99.8	100	100
AB28_Amersfoort5 (A5)	99.4	99.2	99.4	99.7	99.5	99	99.7	98.2	99.5	99.8	99.8	99.8	100	100
AB28_Oordrag1 (O1)	99.4	99.2	99.4	99.7	99.5	99	99.7	98.2	99.5	99.8	99.8	99.8	100	100
AB28_Amersfoort2 (A2)	99.5	99.4	99.5	99.8	99.7	99.2	99.8	98.4	99.7	100	100	100	100	100
AB28_Bethal (B)	99.2	99	99.2	99.5	99.4	98.9	99.5	98.1	99.4	99.7	99.7	99.7	100	100
AB28_Amersfoort6 (A6)	99.5	99.4	99.5	99.8	99.7	99.2	99.8	98.4	99.7	100	100	100	100	99.5
Aa (G)[3]	84.7	84.6	84.7	85.1	85.1	84.9	84.9	84.3	85	85.2	85.2	85.3	85.8	85.4
TW81_Aa (S)[4]	84.5	84.5	84.5	84.9	84.8	84.6	84.7	84.1	84.7	84.9	85	85	85	84.6
AB28_Aa (S)[5]	84.6	84.5	84.6	85	85	84.8	84.8	84.2	84.9	85.1	85.1	85.2	85.6	85.3

[1]TW81_Sw (R) - TW81 *Subanguina wevelli* (GCI-reference)

[2]AB28_Sw (R) - AB28 *Subanguina wevelli* (GCI-reference)

[3]Aa (G) - *Anguina agrostis* (GenBank-reference)

[4]TW81_Aa (S) - TW81 *Anguina agrostis* (Subbotin-reference)

[5]AB28_Aa (S) - AB28 *Anguina agrostis* (Subbotin-reference)

Table 1: Percentage similarities between the SLGN populations from 12 localities as well as *Subanguina wevelli* (GCI-reference) and *Anguina agrostis* (GenBank- and Subbotin-reference) (continues).

TW81_Amersfoort2 (A2)	AB28 A4	AB28 P	AB28 A8	AB28 Sw(R)	AB28 A3	AB28 A7	AB28 A5	AB28 O1	AB28 A2	AB28 B	AB28 A6	Aa(G)	TW81 Aa(S)	AB28 Aa(S)
TW81_Amersfoort5 (A5)	99.2	99.4	99.4	99.5	99.5	99.4	99.4	99.4	99.5	99.2	99.5	84.7	84.5	84.6
TW81_Amersfoort7 (A7)	99	99.2	99.2	99.4	99.4	99.2	99.2	99.2	99.4	99	99.4	84.6	84.5	84.5
TW81_Amersfoort3 (A3)	99.2	99.4	99 4	99.5	99.5	99.4	99.4	99.4	99.5	99.2	99.5	84.7	84.5	84.6
TW81_Oordrag1 (O1)	99.5	99.7	99.7	99.8	99.8	99.7	99.7	99.7	99.8	99.5	99.8	85.1	84.9	85
TW81_Amersfoort8 (A8)	99.4	99.5	99.5	99.7	99.7	99.5	99.5	99.5	99.7	99.4	99.7	85.1	84.8	85
TW81_Bethal (B)	98.9	99	99	99.2	99.2	99	99	99	99.2	98.9	99.2	84.9	84.6	84.8
TW81_Amersfoort6 (A6)	99.5	99.7	99.7	99.8	99.8	99.7	99 7	99.7	99.8	99.5	99.8	84.9	84.7	84.8
TW81_Sw (R)[1]	98.1	98.2	98.2	98.4	98.4	98.2	98.2	98.2	98.4	98.1	98.4	84.3	84.1	84.2
TW81_Amersfoort1 (A1)	99.4	99.5	99.5	99.7	99.7	99.5	99.5	99.5	99.7	99.4	99.7	85	84.7	84.9
TW81_Amersfoort9 (A9)	99.7	99.8	99.8	100	100	99.8	99.8	99.8	100	99.7	100	85.2	84.9	85.1
TW81_Potchefstroom (P)	99.7	99.8	99 8	100	100	99.8	99.8	99.8	100	99.7	100	85.2	85	85.1
AB28_Amersfoort1 (A1)	99.7	99.8	99.8	100	100	99.8	99.8	99.8	100	99.7	100	85.3	85	85.2
AB28_Amersfoort9 (A9)	100	100	100	100	100	100	100	100	100	100	100	85.8	85	85.6
AB28_Amersfoort4 (A4)	100	100	100	100	100	100	100	100	100	100	99.5	85.4	84.6	85.3
AB28_Potchefstroom (P)	100	100	100	100	100	100	100	100	100	100	100	85.7	85	85.6
AB28_Amersfoort8 (A8)	100	100	100	100	100	100	100	100	100	100	100	85.8	85.1	85.6
AB28_Sw (R)[2]	100	100	100	100	100	100	100	100	100	100	100	85.8	85.1	85.7
AB28_Amersfoort3 (A3)	100	100	100	100	100	100	100	100	100	99.8	100	85.9	85.2	85.6
AB28_Amersfoort7 (A7)	100	100	100	100	100	100	100	100	100	99.8	100	85.8	85.1	85.5
AB28_Amersfoort5 (A5)	100	100	100	100	100	100	100	100	100	99.8	100	85.8	85.1	85.6
AB28_Oordrag1 (O1)	100	100	100	100	100	100	100	100	100	100	100	85.8	85.1	85.6
AB28_Amersfoort2 (A2)	100	100	100	100	100	100	100	100	100	100	100	85.8	85.1	85.6
AB28_Bethal (B)	100	100	100	100	100	100	100	100	100	100	100	85.9	85.2	85.6
AB28_Amersfoort6 (A6)	100	100	100	99 8	99 8	99 8	100	100	100	100	99.7	85.6	84.9	85.3
Aa (G)[3]	100	100	100	100	100	100	100	100	100	99.7	100	85.8	85.1	85.5
TW81_Aa (S)[4]	85.7	85.8	85.8	85.9	85.8	85.8	85.8	85.8	85.9	85.6	85.8	100	99.4	99.7
AB28_Aa (S)[5]	85	85.1	85.1	85.2	85.1	85.1	85.1	85.1	85.2	84.9	85.1	99.4	100	99.7
AB28_Aa (S)[5]	85.6	85.6	85.7	85.6	85.5	85.6	85.6	85.6	85.6	85.3	85.5	99.7	99.7	100

[1]TW81_Sw (R) - TW81 *Subanguina wevelli* (GCI-reference)

[2]AB28_Sw (R) - AB28 *Subanguina wevelli* (GCI-reference)

[3]Aa (G) - *Anguina agrostis* (GenBank-reference)

[4]TW81_Aa (S) - TW81 *Anguina agrostis* (Subbotin-reference)

[5]AB28_Aa (S) - AB28 *Anguina agrostis* (Subbotin-reference)