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Agnes Jonsson

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Agnes Jonsson

Supervisor: Maria Viketoft , SLU, Department of Ecology
External supervisor: Maria Schück, Incotec Sweden AB

Examiner: Jonathan Yuen, SLU, Department of Forest Mycology and Plant Pathology

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Abstract

A commonly used forage grass in Brazil is *Brachiaria* spp. These grasses are used as feed for beef and cattle production and are grown on approximately 70 million hectares in Brazil. A pasture with *Brachiaria* spp. can last for 20 years and is relatively free from diseases. Two of the pathogens that do attack *Brachiaria* spp. are the nematodes *Aphelenchoides besseyi* and *Ditylenchus dipsaci*. These nematodes are seed-borne and affect the host plant by eating of the plants growing parts of stems and leaves. As the plant matures, the nematodes move to the seeds where they overwinter or stay until the seeds get in contact with moisture. Both *A. besseyi* and *D. dipsaci* can survive in the seeds in a dry state for several years. Both of these nematodes are classified as quarantine pests due to the damage they cause on their host plants and economically. To stop the spreading of *A. besseyi* and *D. dipsaci*, chemical or hot water treatments are used to control the nematodes. In Sweden, a company called Incotec Sweden AB has developed a method for treating seed-borne diseases, named ThermoSeed. This treats the seeds with different intensities of steam. To evaluate their treatment, a method to extract nematodes from *Brachiaria* seed is needed to be able to guarantee seeds without living nematodes. The purpose of this master thesis was to find a method for extracting nematodes from *Brachiaria ruzizensis* seed that is fast, reliable and cost effective. Four different methods were tested: the Baermann funnel method, a method developed by Zuckermann et al., a method developed by Coolen and D'Herde and The simple method developed by Hoshino and Togashi. To test the methods, three types of treated seeds were used. Untreated seed (positive control), treated seeds with Incotec treatment and negative control (seeds that have been treated multiple times with high steam intensity). The methods were analyzed by counting the recovered nematodes from each of the methods. The data collected were analyzed statistically with ANOVA and a following post hoc Tukey test. From these analyses it was concluded that the Baermann funnel was the method which extracted the most nematodes from the untreated seeds compared to the other methods. The Baermann funnel method also gave clear samples and was easy to conduct.

Keywords: Aphelenchoides besseyi, Ditylenchus dipsaci, Brachiaria ruzizensis, seed treatment, ThermoSeed

Sammanfattning

Ett vanligt förekommande fodergräs i Brasilien är släktet *Brachiaria* spp. Detta gräs odlas idag på ca 70 miljoner hektar vilket kan sättas i perspektiv med Sveriges totala jordbruksmark som är ca 2.6 miljoner hektar. En vall med *Brachiaria* kan leva i upp till 20 år och behöver varken mycket gödsling eller vatten för att överleva. Det är ett relativt friskt gräs med få sjukdomar. Två av de patogener som dock kan angripa *Brachiaria* är de växtpatogena nematoderna *Aphelenchoides besseyi* och *Ditylenchus dipsaci*. Dessa nematoder är fröburna och påverkar värdväxten genom att äta av växtens växande delar av stammar och blad. När växten mognar förflyttar sig nematoderna till fröna och stannar där tills fröna har mognat. Både *A. besseyi* och *D. dipsaci* kan överleva i ett torkat stadie i fröna i ett flertal år. När fröna kommer i kontakt med fukt återfuktas även nematoderna som sedan börjar äta av växtens groende delar. Detta påverkar frönas grobarhet och föryngring av vallen. Dessa nematoder bedöms göra så stor åverkan på dess värdväxter och ur en ekonomisk aspekt att de klassas som karantänskadegörare. För att förhindra spridning av dessa nematoder behandlas idag fröerna kemiskt eller genom varmvattenbehandling. I Sverige har företaget Incotec Sweden AB utvecklat en metod att behandla utsädesburna sjukdomar som kallas ThermoSeed. Denna behandling går ut på att behandla frön med olika intensitet av ånga. För att kunna veta hur bra behandlingen funkar behövs en metod för att driva ut eventuella levande nematoder ur fröna.

Detta masterarbete hade som mål att hitta en metod för att driva ut nematoder ur *Brachiara* frön på ett enkelt, snabbt och pålitligt sätt. I detta arbete testades fyra olika metoder för utdrivning av nematoder med ett obehandlat fröparti, ett behandlat av Incotec och ett parti som garanterat inte innehåller några levande nematoder (negativ kontroll). De fyra metoderna som testades var Baermann- trattar, en metod utvecklad av Zuckermann et al., en metod utvecklad av Coolen och D'Herde och "The simple method" utvecklad av Hoshino och Tagashi. Metoderna analyserades genom att räkna hur många nematoder totalt som drivats ut med respektive metod. Därefter jämfördes metoderna statistiskt med ANOVA följt av Tukey post hoc analys. Från analyserna kunde konstateras att Baermann-trattmetoden skiljde sig signifikant från de andra metoderna med fler utdrivna nematoder än de andra. Det var även denna metod som gav klarast prov och därför var enklast att analysera. Som slutsats kan konstateras att för detta syfte, att hitta en metod som snabbt, enkelt och pålitligt driver ut nematoder, är Baermann-tratt metoden den av de testade metoderna bästa.

Nyckelord: Aphelenchoides besseyi, Ditylenchus dipsaci, Brachiaria ruzizensis, fröbehandling, ThermoSeed

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1 Introduction

Brazil is one of the world leading countries of cattle and beef production. Brazil also has large areas of rainforest that are threatened by an increasing need of agricultural land to sustain the needs of products like sugar cane, corn and cattle production. These needs have led to deforestation of rainforest and affect the ecosystems and biotopes existing there. Deforestation of the rainforest in Brazil is performed by cutting and burning which releases the carbon stored. The rainforest is a large reservoir of carbon that is released to the atmosphere as greenhouse gases when the rainforest is deforested (Nepstad et al., 2008). A mean to prevent deforestation is to make sure that the land is used as efficiently as possible. In 2011, 2 753 730 km^2 of Brazil's total land area (85 157 770 km^2) were used as agricultural land. Of the agricultural land, 23% is used for permanent meadows and pastures (FAOSTAT, 2014). For comparison, Sweden has approximately 31 000 km^2 agricultural land (SCB, 2010). A common grass genus used in Brazilian pastures is *Brachiaria* spp. which is grown on approximately 300 000 - 700 000 km^2 (Miles et al., 1996). These grasses are attacked by the plant-pathogenic nematodes, *Aphelenchoides besseyi* and *Ditylenchus dipsaci* which are seed-borne. According to Favoreto et al. (2006) among others, the nematodes affect the ability to sell the *Brachiaria* seeds and the durability of the pastures. Due to the severe damage these plant-pathogenic nematodes cause, both to the plants and also economically, they are classified as quarantine pests (Agrios, 2005; Capinera, 2008). According to Marchi et al. (2007) the plant-pathogenic nematodes have spread due to poor sanitary conditions of seed. Exporters and importers of *Brachiaria* seed, wants to establish sanitary restrictions on products from Brazil to stop the spreading of pathogens (Marchi et al., 2007). If the amount of infected seed can be decreased, the pastures can get a longer life-time and keep a high productivity. Therefor it is important to have an effective way to treat the seeds against the nematodes. Today these nematodes are mostly controlled by treating the seeds with chemicals (CABI, 2015). Methods using hot water treatment on plants and seeds of other plants,

for example rice, onion and strawberries, are used and can be effective for both *A. besseyi* and *D. dipsaci* (CABI & EPPO n.d.a ; CABI & EPPO n.d.b.).

1.1 Purpose and aim

A new method for treating nematode infested *Brachiaria* seeds is being developed by Incotec Sweden AB, Uppsala. To be able to assure that the seeds are free from living nematodes after treatment, a method for control of the treated seeds is needed. This method needs to be able to separate living nematodes from dead since the treatment method leaves the dead nematodes on the seed. Many methods used for extracting nematodes only measure whether nematodes are present in the seeds or not and they cannot distinguish between living and dead nematodes. The purpose of this thesis was to find a method that can be used for detecting living nematodes of *A. besseyi* and *D. dipsaci* in *Brachiaria* seeds. Methods where no living nematodes can be detected will not be tested and this thesis will not include testing of seed vitality. The aim was to find a method that detects living nematodes and in addition is reliable, easy to use and time efficient. The thesis has been carried out as a practical comparative study and a literature review.

2 Literature review

In this chapter the host plant *Brachiaria*, the nematodes *D. dipsaci* and *A. besseyi*, and the methods to extract nematodes from seeds are described.

2.1 The host plant *Brachiaria*

Brachiaria is a genus of grass that often is used as feed for livestock. The genus contains about 100 species and is native to tropical Africa. There are both annual and perennial species and all species are C4 carbon fixators which means that their photorespiration is lower than in C3 plants (Campbell et al., 2008).This makes *Brachiaria* stand drought and high temperatures better than most other plants. Since the genus contains a large number of species, not all have the same features. The majority spread by seeds but some species spread by rhizomes, and some species can grow more than 1.5 meters tall while some species grows less than 1 meter tall (Skerman & Riveros 1990). Most *Brachiaria* species can be heavily grazed and many can form dense swards. Many species also does not require high amounts of fertilizer and can grow well on poor soils (Skerman & Riveros, 1990). Although *Brachiaria* is commonly used as a forage grass for pastures and meadows, it can be toxic to sheep, goats and young cattle and can give fatal damages to the animals (Miles et al., 1996). *Brachiaria decumbens* was the first *Brachiaria* species introduced to Brazil in the 1950's followed by other species such as *B. ruzizensis* (Miles et al., 1996). This introduction was done to improve the pasture production in Brazil. According to Santos et al. (2014), 85% of the cultivated pastures in Brazil contain *Brachiaria* spp. Pastures of *Brachiaria* can last more than 20 years with little or no fertilizer (Miles et al., 1996; Heuzé et al., 2013).

In this thesis, the species *B. ruzizensis* is used for testing the methods but the methods should work for other species of *Brachiaria*. At the beginning of the practical study, there were two different seed batches to choose from, one batch of *B. ruzizensis* and one batch of *B. brizantha*. *Brachiaria ruzizensis* was chosen because this batch contained more seeds and could be used for a larger number of tests and repetitions.

2.1.1 Brachiaria ruzizensis

The species *B. ruzizensis* is perennial and can spread with short rhizomes and seeds (Skerman & Riveros, 1990). It has good drought tolerance (FAO.org n.d.) and has a high nutrient content (Skerman & Riveros, 1990). To grow well, it needs fertile and drained soils. It can grow as high as 1.5 meters and has an optimal growing temperature between 28-30° C (Skerman & Riveros 1990; Tropicalforages.info 2005). The seeds are flat with a pear-like shape and are about 5 mm long and 1- 3 mm wide, see figure 1.

Brachiaria ruzizensis seeds, like many *Brachiaria* species, have a physiological dormancy that has to be broken for the seeds to germinate (Filho & Usberti, 2008). This dormancy can be broken by treating the seeds with acid. Different methods of breaking the dormancy have been tested including hot water treatment (85 °C) and sulfuric acid treatment (Montório et al., 2008). According to Montório et al. (2008), hot water treatment was not sufficient enough to break the dormancy. CABI & EPPO (n.d) states that the nematode *A. besseyi* can be killed by soaking seed in water for 10 minutes at 50-60 °C. This would mean that the dormancy will not be affected by the treatment for killing the nematodes.

Figure 1. Picture of *Brachiaria ruzizensis* seeds.

2.2 The nematodes

Nematodes belong to the phylum Nematoda and are also called roundworms (Campbell et al., 2008). They have non-segmented, cylindrical bodies and the body size can range between less than 1 mm to over 1 meter in length depending on the species. Soil-living nematodes are in general around 1 mm and it is animal parasites that may reach lengths of several meters. Nematodes usually reproduce sexually (sexes are often separate) but there are species that reproduce parthenogeneticically. Nematode species are found in soil, plants, oceans, and in animals. The nematode species roles range between predators and parasites (Campbell et al., 2008). Nematodes feeding on plants can use their stylet (a needle-like structure in their mouth) to suck out nutritious fluids from the plant cells. Other plant-parasitic nematodes can go inside the plant and either move around inside the plant or make the plant produce a feeding cell where the nematode becomes sedentary (Agrios, 2005).

Here follows a short introduction to the nematodes *A. besseyi* and *D. dipsaci* that are known as pathogens *Brachiaria* spp. These nematodes affect the productivity of pastures by shortening the lifespan of the pastures by feeding on the plants. Nematode infested seeds will also have a delayed germination, causing problems at the establishment of new pastures (Favoreto et al., 2011).

2.2.1 *Ditylenchus dipsaci* (Kühn) Filipjev

Ditylenchus dipsaci, also known as the stem and bulb nematode, is found world-wide and can attack a large variation of hosts. *Ditylenchus dipsaci* have more than 450 host plants (CABI & EPPO n.d.b). According to CABI & EPPO (n.d.b) ten biological races have been reported, some very host specific *e.g.* the strawberry race which only have strawberries as a main host and very few alternative host plant species. Unfortunately, it is not known what race of *D. dipsaci* that attacks *Brachiaria*. *Ditylenchus dipsaci* is most destructive in temperate climate (Agrios, 2005), where it causes dwarfing of plants, swollen and twisted stems, reduced yields and killing of seedlings. In *Brachiaria, D. dipsaci* cause swelling and de-forming of leaves and stems, and necrosis or rotting of stem bases.

Ditylenchus dipsaci is 1 – 1.3 mm long and about 30 µm in width (Agrios, 2005). Females lay 200 – 500 eggs that are often fertilized by males (CABI & EPPO, n.d.b; Agrios, 2005). Depending on the stage of the plant, *D. dipsaci* enters the plant in different ways. In young plants, entrance happens through the stomata of leaves or through direct penetration of the leaves. In seedlings and germinating seeds, *D. dipsaci* enters at the root cap or at parts that are germinating from the seed (Agrios, 2005). The nematodes remain in the intercellular spaces and feed from parenchyma cells. Reproduction also takes place in the intercellular spaces (Agrios, 2005). Nematodes extracted from dry plant material are likely to be stage 4 juveniles (Starr et al., 2002) because the stage 4 juveniles migrate to the seeds before the seeds have matured. The nematodes dry out together with the seeds, a phenomena that is called cryptobiosis. When the seeds then are rehydrated, the nematodes also get rehydrated and will be the same stage as before dehydrating. If the number of nematodes increases, the fourth stage juveniles aggregate and form clumps of "eelworm wool" which can survive many years if the conditions are dry. This "eelworm wool" is formed on or just below the surface of infested plant tissue and can attach to the seeds of the host plant (CABI & EPPO, n.d.b).

The optimal temperature for activity and invasion is 10 - 20° C (CABI, 2015). The life cycle takes about 20 days at 15° C in onion, but the length of the life cycle depends on temperature and also differs between races and isolates (CABI, 2015).

Ditylenchus dipsaci, as a pest in *Brachiaria*, was first reported in South America in 1929 (CABI, 2015). Today *D. dipsaci* is found in six parts of Brazil: Minas Gerais, Paraiba, Parana, Rio Grande do Sul, Santa Catarina, Sao Paulo (CABI & EPPO, 2009).

2.2.2 *Aphelenchoides besseyi* Christie

Aphelencoides besseyi is a seed-borne nematode species that can survive on dried seeds for more than three years. Although it can survive for a relatively long time on dried seeds, it will not survive in the soil without a host plant. *Aphelenchoides besseyi* has many different hosts, but rice and strawberries are the most important ones. Other hosts are ornamental plants and grasses such as *Brachiaria* spp. In rice, *A. besseyi* causes white tips of the leaves and in strawberries the leaves can get wrinkly and/or the plants can become dwarfed (CABI & EPPO, n.d.a). After seeds are sown and germinate, *A. besseyi* becomes active and starts to move to the growing points of the crop. At the growing points of leaves and stems, the nematodes start feeding on these parts ectoparasitically. When the plant has matured, in comparison to *D. dipsaci* who stays on the outside of the seed, *A. besseyi* coils up inside the palea but never goes inside the seed. According to CABI & EPPO (n.d.a) up to 14 nematodes can be found in one rice seed.

The optimal temperature for development of *A. besseyi* is 21-25° C (Luc et al., 2005). *Aphelenchoides besseyi* has a shorter and slender body compared to *D. dipsaci*. The length of the body ranges between 0.44 and 0.84 mm, and the width between 14 and 22 µm (CABI & EPPO, n.d.a). The lifecycle of *A. besseyi* takes 8-10 days depending on the temperature. According to CABI & EPPO (n.d.a) the cycle takes 10 days at 21° C. However, Luc et al. (2005) states that the optimal temperature is 30° C and the life cycle then takes 10 ± 2 days. This would mean that *A. besseyi* can reproduce in the plant and move to the seeds for overwintering. According to Ferris (2013), no development occurs below 13° C and optimal temperature for hatching and egg laying is 30 ° C. For reproduction, fertilization is not needed as *A. besseyi* can reproduce parthenogenetically, which means that an embryo can form from an unfertilized egg cell (CABI & EPPO n.d.a).

2.3 Methods for nematode detection in seeds

In this section, ten methods found in the literature review will be described. There are three different principles of extracting nematodes that can be combined with each other (EPPO Bulletin, 2013). The first principle is motility of the nematodes, meaning that the nematodes actively move from the sample to water. The second principle is size and shape of the nematodes, meaning that nematodes are collected on sieves by passing samples. The third and last principle is the densities of the nematodes, meaning that nematodes are separated from samples by centrifuging or floating (EPPO Bulletin, 2013). Nine of the ten methods found depend on movement of the nematodes. The one method that does not depend on movement of the nematodes is the Coolen and D'Herde method. This method crushes the seeds and

then centrifuges them which separate the nematodes from the samples by different sedimentation rates.

The Zuckermann et al method (3) is the only method developed to extract nematodes from *Brachiaria* seed. Five of the ten methods are developed for other types of seeds *e.g.* rice. Those methods are the Dehulled seed method (4), The simple extraction method (5), Baermann funnel by Hoshino and Togashi (6), the "EPPO" method (9) and the method by Augustin and Sikora (10). The Modified-tray technique method (7) is developed for onions, and the Baermann funnel method (1) and Oostenbrink dish method (2) can be used for soil samples or any plant tissue. The Coolen and D'Herde method (8) is developed for root samples containing swollen nematodes attached to the roots.

The methods have been developed for *A. besseyi*, *D. dipsaci* or both. Methods 1, 2 and 8 are used for extraction of both *A. besseyi* and *D. dipsaci*. Methods 3, 4, 5, 6 and 9 are used for *A. besseyi*, while methods 7 and 10 are used for *D. dipsaci*. In this thesis, all methods are tested on seed infected with both *A. besseyi* and *D. dipsaci*.

1. The Baermann funnel method

Soil or plant tissue is placed in a funnel that holds a sieve and a filter paper. The funnel end is covered with rubber tubing with a clamp at the bottom. Water is added to cover the soil or plant tissue. Nematodes move through the filter paper and wire screen and sinks to the bottom. After 24 -48 hours nematodes are collected by emptying 5 -8 ml of water by removing the clamp from the rubber tubing in a shallow dish (Agrios, 2005). Originally the Baermann funnel method used a cloth instead of a sieve and filter paper. The cloth was almost completely covered in water and resulted in low nematode recovery (EPPO, 2013).

2. Oostenbrink dish method

This method is derived from the Baermann funnel method and is based on the same principle that the nematodes move from the sample to the water. Instead of using a funnel, a dish is used. Thus soil or plant tissue is placed on a wire sieve with filter paper or a fabric with fine mesh on it. These parts are placed in a dish and water is added. After a few days, the water from the dish is collected which contains the nematodes (EPPO, 2013).

3. The Zuckermann et al (1990) method (from Tenente et al., 2006)

Seeds (*Brachiaria*) are soaked in distilled water for 16 hours. The samples are then poured into a blender and blended for 20 seconds. The blended suspension is rinsed on a sieve with a mesh with 0.025 mm pores and the sieve is placed on a Baermann funnel for 24-72 hours. Nematodes that have passed the mesh are counted with a dissecting microscope (Tenente et al., 2006).

4. Dehulled seed method

Dehull seeds (rice) with a mill with 1 mm distance between the rolls. The dehulled seeds are put on a nylon sieve with a 0.25 mm mesh fitted on a 45 mm diameter beaker. The beaker is filled with 20 ml of water. Samples are left for 24 hours at 25 +/- 2° C, then the sieve is removed and the water collected for microscopic examination (ISTA, 2014).

5. "The Simple extraction method" by Hoshino and Togashi (1999)

Seeds (rice), cut longitudinally in two, are placed in single plastic pipette tips. Each single pipette tip is placed in a glass vial with water. Samples are left for 24 hours at 25° C in dark. If the rate of the nematode extraction is examined, move the pipette tips to new glass vials with water at 2, 4, 8 and 24 hours after start of extraction. Water samples are then transferred to Syracuse watch glasses for counting of both living and dead nematodes. Nematodes that do not move when a needle is pushed against the nematode are considered dead (Hoshino & Togashi, 1999).

6. Baermann funnel technique described by Hoshino and Togashi (1999)

Fifty seeds (rice), cut in two, are placed on a Japanese paper (Improved paper 17, Heiwa Shigyou, Osaka, Japan). The Japanese paper is soaked in water in a Baermann funnel with 9 cm in diameter and then left for 24 hours at 25° C in darkness. Nematodes, both living and dead, are collected from the funnel bottom, inner funnel wall and from the seeds (Hoshino & Togashi, 1999).

7. Modified-tray technique by Whitehead and Hemming (1965) from Tenente & Evans (1993)

Plant samples are placed on small trays with a plastic mesh and paper. Distilled water is added to wet the plant sample and it is left for 24 hours. The water is collected and passed through a sieve and the content collected by the sieve is transferred to a beaker and the number of nematodes is counted. New water is added to

the trays and the procedure is repeated at 48 and 72 hours (Tenente & Evans, 1993).

8. The Coolen and D'Herde (1972) method

Seeds, that may have been rehydrated, are grinded, sieved and washed. Kaolin is added to the mixture which is then centrifuged. After centrifugation, the pellet is re-suspended with a sugar solution and centrifuged again. The nematodes in the supernatant are collected with sieves and washed (Coolen & D'Herde 1972; Ministério da agricultura, pecuária e abastecimento secretaria de defesa agropecuária 2009).

9. "EPPO" method

Seeds placed on a nylon sieve are soaked in water in shallow dishes of water. The samples are soaked for 4-5 days at 25 +/- 2° C. The nylon sieve with the seed samples is removed every day to collect the water for microscopic examination. The dishes are refilled with fresh water and the nylon sieve with the samples put back in place. The nylon sieve is made of a nylon material with a pore size of 0.25 mm that is glued to a cylindrical tube. Approximately 100 seed (rice) are used (International seed testing Association 2008).

10. Method from Augustin and Sikora (1989)

Seeds are submerged in water for 24 hours at 10° C. The water is then passed on a sieve and the nematodes collected on the sieve are counted (CABI, 2015).

After extraction, regardless of method, the samples are analyzed with a dissecting microscope and the nematodes recovered are counted.

A number of methods have been found in this literature review. According to EPPO (2013), the best methods for extraction of *A. besseyi* and *D. dipsaci* from seeds are the Baermann funnel and the Oostenbrink dish. Favoreto et al. (2006) have compared methods of nematode extraction from seeds of *B. brizantha*. The methods compared were variations of Coolen & D'Herde (1972) and Baermann funnel methods. They compared six different seed treatments. The first treatment was with seeds that had not been rehydrated which were tested with Coolen & D'Herde (1972). The second treatment was seeds that had been rehydrated and which also were tested with Coolen & D'Herde (1972). The remaining four treatments were whole seeds, whole rehydrated seeds, seeds that had been grounded and rehydrated seeds that had been grounded. These treatments were tested with the Baermann funnel method. After comparison, the best method was the Coolen & D'Herde method with seeds that had not been rehydrated. Favoreto et al. (2006)

defined the best method as the method with most recovered nematodes. This study by Favoreto et al. (2006) have resulted in that the Brazilian ministry of agriculture recommend to use the Coolen and D'herde method for extraction of *A. besseyi* and *D. dipsaci* from *Brachiaria* seeds (Ministério da agricultura, pecuária e abastecimento secretaria de defesa agropecuária, 2009).

2.4 Distinguishing between dead and alive nematodes

To know how successful the seed treatments are, a way of distinguishing if the extracted nematodes are dead or alive is needed. One way to do this is to poke each nematode with a needle, and if the nematode reacts to the needle it is alive and if it does not react it is considered to be dead. This method has some drawbacks, one is that every nematode needs to be poked and examined which is time consuming. Another drawback is that *D. dipsaci* is known to have a dormancy state in which it would not react to the needle. The advantage is that it is a relatively cheap and simple method to use.

Instead of using a needle, there are methods of staining that are able to differentiate between living and dead tissue. Some methods use florescent staining, others are based on colors (Jatala, 1975; Bird, 1979). The use of a staining method could make it possible to quickly determine if there are any living nematodes. These methods would not have the same problems with *D. dipsaci* and its possible dormancy. Furthermore, these methods would give a fast answer to if the nematodes are dead or alive and each nematode would not be needed to examine. The problems with these methods are that some of the staining compounds are toxic and that some methods *e.g.* florescent methods, need special equipment to see the florescent light. Another problem is that it is not very clear how well these methods actually work on nematodes.

Another method for determining if the nematodes are living or dead is to use NaOH. By putting NaOH in the water samples with nematodes, the living ones goes from straight to a j-shape while the dead ones remain straight (Chen & Dickson, 2000).

3 Material and methods

This chapter contains description of the tested extracting methods and staining methods. The seeds and the different seed treatments used are described.

3.1 Extraction methods

From the methods listed above, four methods were chosen for testing. These four methods were method no. 1 The Baermann funnel method, 3 The Zuckermann et al method, 5 The simple extraction method and 8 The Coolen and D'Herde method.

The reasons for choosing these methods are their principle of extraction, how easy they are to perform, that they use different types of equipment, and different handling of the seeds prior to extraction. Methods 1, 2, 4, 5, 6, 7, 9 and 10 are based on the same principle that living nematodes can move from the sample to the water. Due to the similarities of all these methods, only method 1 and method 5 were chosen to be tested. Method no. 3 also uses the Baermann funnel but have a step with blending the seeds before putting it on the funnel. Method number 5 uses pipette tips to hold the seeds and the nematodes swim out from the tip. This gives the advantage of clean and easy to analyze samples. It is also a method that uses different materials than methods using funnels. Method number 8 uses a completely different method than the other chosen methods. This method uses a centrifuge to separate the nematodes from the seeds, thus based on the principle of gravity.

Stacks used for the funnel extractions were made to keep the funnels steady and still and also to be stable when handling the funnels. The stacks used for the Baermann funnels were two net baskets made out of metal turned upside-down and then four legs (plastic pipes) for each basket was attached by straps (Figure 2a). The stacks

Figure 2. Stacks of A) Baermann funnels and B) Zuckermann et al method.

used for the Zuckermann et al method were made by drilling holes in a wooden board (Figure 2b).

To compare the different extraction methods, the nematode samples were placed in a counting dish and the number of nematodes was counted using a dissecting microscope at 25 magnifications.

After the tests ended, all materials used were sterilized (autoclaved or washed in 70% ethanol) or sent to destruction.

3.1.1 Baermann funnel

Sieves were made of plastic pipes with the outer diameter 10 cm that were cut in 4-5 cm sections. These sections were cleaned with water and with 70 % ethanol prior to the start of the experiment. Net pieces (15 X14 cm) with pore size of 1 mm were fixed on the pipe sections with rubber bands creating a level surface. One piece of Vliseline (14x14 cm) was put into each sieve. Rubber tubing was put on the funnels and clamps were put on the rubber tubes and the funnels were put in the stacks.

5 replicates of 5 g of *Brachiaria* seeds of each of the three different seed treatments were measured and put into the sieves. The sieves were put into the funnels and distilled water was added to the funnels passing the sieves, bringing the water level up to the sieve but not so that the water completely covered the seed samples. The different treatments were randomly distributed on the stacks. Nematode samples were taken from the funnels every second day by emptying 4-6 ml of the water by relaxing the clamps in the end of the rubber tubing. Water was refilled after samples were taken.

This procedure was repeated once so that each seed treatment was tested 10 times.

3.1.2 Zuckermann et al method

The making of the sieves was the same procedure as for the Baermann funnel method with the only differences being the mesh size and that filter paper were used instead of Vliseline. Sieves were made of plastic pipes with the outer diameter 10 cm that were cut in 4-5 cm sections. These sections were then cleaned with water and with 70 % ethanol. Then, net with the pore size of 40 µm was cut in pieces (15 x 14 cm). The net pieces were fixed on the pipe sections with rubber bands creating a level surface, and filter paper was put into each sieve. Rubber tubing was put on the funnels and clamps were put on the rubber tubes and the funnels were put in the stacks.

5 replicates of 5g of *Brachiara* seeds of the three different seed treatments were put in small petri dishes and soaked in water for about 16 hours. The samples and the water were then crushed together by mortar and put in the sieves in the funnels. Distilled water was added to the funnels passing the sieves, bringing the water level up to the sieve but not so that the water completely covered the seed samples. The different treatments were randomly distributed on the stacks. Nematode samples of 4- 6 ml were taken from the funnels every second day. Water was refilled after samples were taken.

This procedure was repeated once so that each seed treatment was tested in total 10 times.

3.1.3 Coolen and D'Herde method

5 grams of seeds were mixed with 20 ml water with a mortar until all the seeds were broken apart. The mixture was poured over a sieve with 1 mm pores and a sieve with 10 µm pores. The seeds that were stuck in the 1 mm sieve were rinsed with more water, which also was passed through the 10µm sieve. When all water had passed through the sieves, the 10 µm sieve was rinsed with water that was collected in a centrifuging tube. About 13 ml of water was collected. Then 0.325 grams of kaolin was added to the centrifuging tube and thoroughly mixed by vortex and with glass rods. The tube was centrifuged at 1800 g for 4 minutes. The centrifuge used was a fixed angle rotor centrifuge called Heraeus Biofuge primo r. After centrifuging the supernatant was poured off and a sucrose solution was added to the tube. The sucrose solution had a density of 1.15 g/cm^3 and was prepared by weighing 35 grams of sucrose and adding 60 ml of water. This solution was then stirred so that the sucrose dissolved in the water. The solution was put on a scale and more water was added until it reached 100 grams. After adding the sucrose solution in the tube, the pellet was mixed with the solution and vertically

shaken. The tube was centrifuged again at 1800 g for 4 minutes. After centrifugation the supernatant was poured over a 10 µm sieve that was rinsed with water and collected. This water was then analyzed with a dissecting microscope.

Kaolin was added to the samples to help the nematodes sink to the pellet during centrifugation. The kaolin forms a net structure in the sample that catches the nematodes and brings them down to the bottom (Coolen & D'Herde, 1972). After centrifugation with the kaolin, another centrifugation takes place but this time with a sucrose solution. The sucrose solution has a density of 1.15 g/cm^3 to make the kaolin sink and form a pellet but to keep the nematodes in the supernatant.

Crushing of the seeds was done by mortar. However, three other tools were tested for the crushing because the blender recommended by Coolen and D'herde (1972) were not owned by Incotec. The first tool tested was a domestic blender, the second an Ultra Turax mixer and the third was a coffee grinder. All of the different methods were done with seeds and water. After examination of the mixtures done by the three stated mixers no nematodes could be found. From previous experience, the mortar was therefore chosen for the mixing.

3.1.4 The simple method

This method was only tested in a small scale with two different amounts of seeds in each pipette tip to evaluate if it was as effective as stated by Hoshino and Togashi (1999). At first, it was planned to test ten vials with one seed in each and 5 vials with two seeds in each to test an equal amount of seeds. Due to human error, nine vials with one seed and six vials of two seeds were tested.

Test with one seed in each pipette tip

Pipette tips were put into nine glass vials. Nine infected and untreated *Brachiaria* seeds were cut in halves. Two halves were put into each pipet tip and 2-3 ml of water was added through the tip. The water was analyzed the next day and then two more times with two days in between. New water added after each analysis.

Test with two seeds in each pipette tip

Pipette tips were put into six glass vials. 12 infected and untreated *Brachiaria* seeds were cut in halves. Four halves were put into each pipette tip and 2-3 ml of water was added through the tip. The water was analyzed the next day and then two more times with two days in between.

3.2 Seeds

Infected *Brachiaria* seeds subjected to three different treatments were used. The treatments were: untreated seeds (positive control), treated seeds with Incotec intensity, and high intensity treated seeds (negative control). The negative control was used to make sure that the methods tested did not extract dead nematodes. The Incotec intensity is a treatment that aims to control the nematodes but not affect the seed quality negatively. The exact intensity used is only known by Incotec. However it is not intense enough to control all nematodes and thus it is expected to find some living ones. The treatments were made by Incotec using their method named ThermoSeed, which treats the seeds with steam.

5 grams of seeds were used in all extraction method tests except for the simple method. After counting 100 *B. ruzizensis* seeds three times, it was concluded that 100 seeds weighed in average 0.95 grams. Due to this, it was decided that 5 grams of seed would be sufficient to test the methods, as it would mean that with ten repetitions of each treatment more than 5000 seeds were tested for each.

The seeds used in this thesis were *B. ruzizensis* seeds due to practical reasons, as it was the *Brachiaria* species that Incotec had adequate amounts of. The seeds had previously been tested by a laboratory in Brazil to establish the infection rate of the seeds using the Coolen and D'Herde method. The infection rate of these seeds was 120 nematodes /10 g seed according to their analysis. This would mean that when testing 5 g of seeds 60 nematodes would presumably be found.

3.3 Staining methods

Here follows descriptions of the three staining methods that were tested. The nematodes for these tests had been extracted from infected untreated *B. ruzizensis* seeds.

Living nematodes were isolated and put in water to a concentration of about 200 nematodes / ml water. 1 ml of this solution was diluted with approximately 20 ml of water. This dilution was then divided into two tubes with about 10 ml in each and one of the tubes was put in boiling water for 20 minutes (Jatala, 1975).

3.3.1 Potassium permanganate (KMnO4)

Two different concentrations of KMnO4 were tested to find out what concentration is needed to stain the nematodes without killing the living ones.

Concentration 0.5%

0.5 ml of Potassium permanganate with the concentration of 1% was added to 0.5 ml of water containing nematodes. This was done with both 0.5 ml of water containing living nematodes and 0.5 ml of water containing dead (boiled) nematodes. The stain was added and after 5 minutes and after 20 hours the nematodes was examined (Jatala, 1975).

Concentration 0.062 %

Potassium permanganate (1%) was added to samples with living and dead (boiled) nematodes so that the final concentration was 0,062 %. The samples were analyzed after 5 minutes and after 20 hours. To be able to analyze the samples, water was added to give the samples clearness (Jatala, 1975).

3.3.2 Florecindiacetate (FDA)

5 mg FDA was mixed with 1 ml of acetone. This mix was then diluted with phosphate buffer with the proportions of 1:25. From this mixture of FDA, acetone and phosphate buffer a drop was taken and put on a nematode sample so that the final concentration was 0.001 %. The nematode sample was then analyzed under a UV-microscope. The rest of the buffer was stored dark and cold in a refrigerator (Bird, 1979).

3.3.3 NaOH –test

1 ml of water containing living nematodes, 1 ml of water containing dead nematodes and one sample containing both living and dead nematodes was put in small petri dishes. 35 µl of 1 M NaOH was put in each petri dish. The samples were analyzed within 2 minutes with a dissecting microscope (Chen & Dickson, 2000).

4 Data treatment and statistical analyses

The total number of nematodes extracted by each method was calculated by summing all the recovered nematodes, living and dead, for each sample in each method and treatment (for data see Appendices A-C). This means that for example there are ten values for the untreated Baermann funnel method and Zuckermann et al method. This gives the results in nematodes per 5 gram seed, to be able to compare the different methods. During the recovery of nematodes the number of dead nematodes found at each analysis was noted. This was done to be able to see how the different methods affected the nematodes after leaving the seeds.

To analyze the results two statistical methods were used; QQ-test and ANOVA followed by Tukey post hoc analysis. The QQ-test determines if there is a linear correlation between two compared methods (Thode 2002) by plotting the quantiles of the samples from one method against the quantiles of the samples from another method. This analysis was done with the data for untreated seeds and was performed in Excel. Differences among the different extractions methods in the number of recovered nematodes were tested with one-way ANOVA followed by Tukey post hoc analysis using R version 3.1.0 (R Core Development Team 2014). In R, the function aov() were used to perform the ANOVA, and the data was log-transformed prior to analysis to meet the assumptions for ANOVA. The different seed treatments were analysed separately.

In the Coolen and D'Herde method, the different crushing results have been compared with each other; untreated seeds; presoaked crushed, presoaked whole, whole and negative control. This has been done visually by plotting the results in a bar chart (see figure 3).

5 Results

In this chapter, the results from the extractions methods and the staining methods are presented. First the results for the extraction methods are presented and then the results for the staining methods.

5.1 Extraction methods

In following sections the results for the four methods tested; The simple method, Coolen and D'Herde method, Baermann funnel method and Zuckermann et al, are presented.

5.1.1 The Simple method

With the Simple method, one nematode was found in one of the nine samples at the first day of analyses. Two days later the vials were analyzed again and two nematodes were found in one of the samples, but not in the same sample as before. When the water of the samples was analyzed the third and last time, no nematodes were found in any of the samples. When two infected and untreated seeds were used in each pipette tip, no nematodes were found in any of the samples. This method was considered not to meet the requirements of Incotec, due to difficulties to test the same amount of seed as the other methods and thus treated seeds were not tested.

5.1.2 Coolen and D'Herde

From visual comparison between A (untreated presoaked crushed), B (untreated presoaked whole) and C (untreated whole) no differences are shown, see Table 1 and Figure 3.

Table 1. Number of nematodes recovered in the repetitions using the Coolen and D'Herde method. A (untreated presoaked crushed seeds), B (untreated presoaked whole seeds), C (untreated whole seeds) and Q (negative control).

	A	B	C	Q
Repetition 1	50	45	18	1
Repetition 2	64	52	23	2
Repetition 3	54	42	70	1

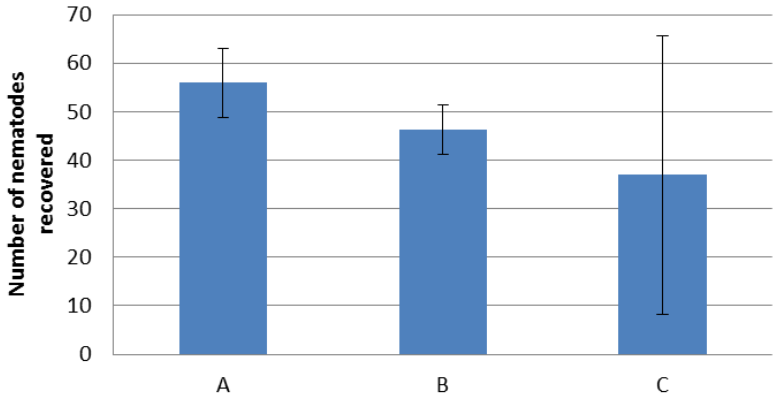

Figure 3. Comparison of mean values and standard deviation for untreated seeds for A (untreated presoaked crushed), B (untreated presoaked whole) and C (untreated whole) for Coolen and D'Herde.

5.1.3 Untreated seeds

The Baermann funnel method (B) was the extraction method that yielded most nematodes (ANOVA: F=12.37, p=0.0003) (Table 2, Figure 4). The numbers of extracted nematodes varied between 26-1147 nematodes/5g seed, with a mean of 448 nematodes/5g seed (Table 2). In total 25 dead nematodes were found in the ten replicates (Table 2).

The Zuckermann et al (Z) and the Coolen and D'Herde (CH,C) method yielded similar numbers of nematodes. With the Zuckermann et al method the largest number of nematodes extracted from the untreated seeds were 110 nematodes/ 5g seed and the lowest number 15 nematodes/5g seed. The mean of the ten repetitions was 50 nematodes/5g seed (see table 2). A total of 74 dead nematodes were found in the samples from the untreated seeds, see table 2. With the Coolen and D'Herde

method the largest number of nematodes extracted from untreated seeds was 70 nematodes/ 5g seed and the lowest number 18 nematodes/5g seed. The mean of the three repetitions was 37nematodes/5g seed (see table 2). No dead nematodes were found in the untreated seeds, see table 2.

Table 2. Number of recovered nematodes for the Baermann funnel method (B), the Zuckermann et al method (Z) and Coolen and D'Herde (CH, C).

Untreated seed									
	B			**Z**			**CH, C**		
	Living	Dead	Total	Living	Dead	Total	Living	Dead	Total
Repetition 1	326	1	327	13	5	18	18	0	18
Repetition 2	135	0	135	15	22	37	23	0	23
Repetition 3	134	1	135	50	25	75	70	0	70
Repetition 4	24	2	26	11	4	15			
Repetition 5	183	2	185	34	11	45			
Repetition 6	1147	0	1147	77	0	77			
Repetition 7	138	0	138	41	0	41			
Repetition 8	689	0	689	23	5	28			
Repetition 9	1008	7	1015	52	0	52			
Repetition 10	669	12	681	110	0	110			
Total	4453	25	4478	426	72	498	111	0	111
Mean values	445,3	2,5	447,8	42,6	7,2	49,8	37	0	37

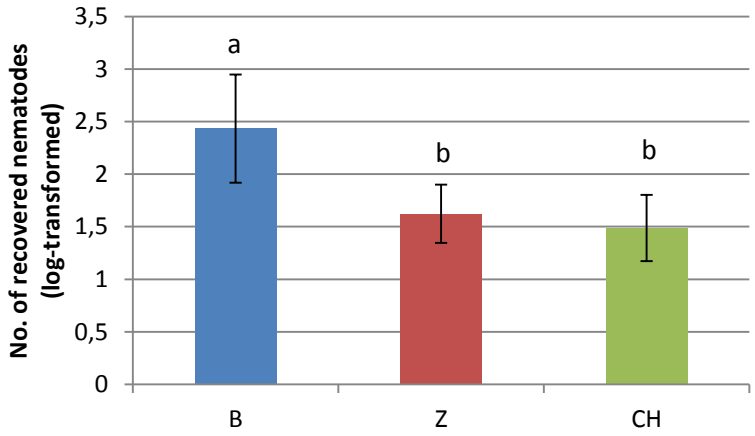

Figure 4. Comparison of transformed mean values and standard deviation of untreated seeds for the three methods, the Baermann funnel method (B), the Zuckermann et al method (Z) and Coolen and D'Herde method (CH). Different letters indicate significant differences ($p<0.05$).

Method B and method Z was compared with a QQ-test, see Figure 5. In this comparison between method B and Z it is shown that there is approximately a linear correlation. This means that the two methods have a similar distribution and differs by a coefficient which in this case is 12,9 (the slope of the line). If the coefficient is known, the results from the Zuckermann et al method could be multiplied with the coefficient and then get similar results as the Baermann funnel method.

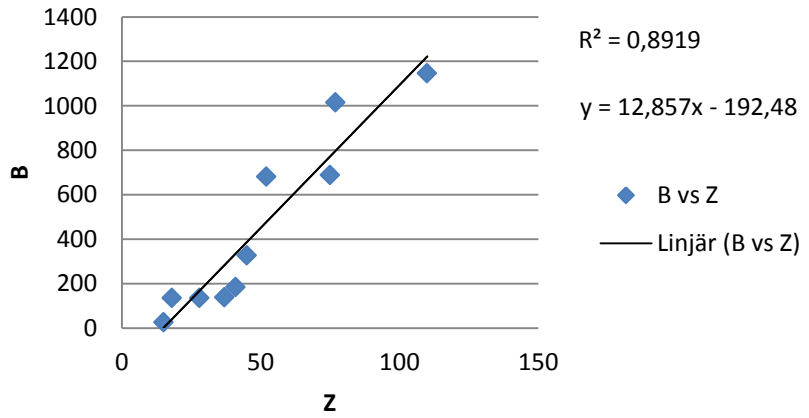

Figure 5. QQ-test comparing the Baermann funnel method (B) with the Zuckermann et al method (Z). The plot shows the distribution of quantiles for the two methods.

5.1.4 Treated seeds

The other treatments, Incotec temperature and negative control, did not show any differences due to that most numbers were 0 (see table 3). The data did not have a normal distribution and therefore no ANOVA or t-test was made.

With the Baermann method, 1 nematode was found in one sample from the seeds treated with Incotec intensity (Table 3). In the negative control replicates, no nematodes were found with this method. With the Coolen and D'Herde method, 4 dead nematodes were found in the negative control replicates (Table 3). The Incotec intensity was not tested with this method due to the recovery of nematodes in the negative control. With the Zuckermann et al method, 4 nematodes were found in seeds treated with Incotec temperature and no nematodes were found in the negative control (Table 3).

Table 3. Number of recovered nematodes for the Baermann funnel method (B), the Zuckermann et al method (Z) and Coolen and D'Herde (CH). With Incotec intensity a total of one nematode was recovered with the Baermann funnel method and four nematodes recovered with the Zuckermann et al method. With the negative control zero nematodes were recovered with B and Z and four nematodes were recovered with CH.

Incotec intensity		
	B	**Z**
Repetition 1	0	0
Repetition 2	1	0
Repetition 3	0	0
Repetition 4	0	0
Repetition 5	0	0
Repetition 6	0	0
Repetition 7	0	1
Repetition 8	0	1
Repetition 9	0	1
Repetition 10	0	1
Mean	0,1	0,4

Neg. Control			
	B	**Z**	**CH**
Repetition 1	0	0	1
Repetition 2	0	0	2
Repetition 3	0	0	1
Repetition 4	0	0	
Repetition 5	0	0	
Repetition 6	0	0	
Repetition 7	0	0	
Repetition 8	0	0	
Repetition 9	0	0	
Repetition 10	0	0	
Mean	0	0	1,333333

5.2 Results of staining

In the following section the results of the tested staining methods are presented.

5.2.1 Potassium permanganate

Both concentrations tested (0.5 % and 0.062%), stained the dead (boiled) nematodes (figure 6) while the living nematodes stayed colorless and started to move. After approximately 20 hours, the dead nematodes still had the same color as after 5 minutes, while the living nematodes could not be found in the sample and seemed to have dissolved. Even with the lower concentration, the living nematodes started reacting to the stain by increased movement and the dead nematodes got a more yellow or orange color.

Figure 6. Dead (boiled) nematode stained by potassium permanganate (0.5 %).

5.2.2 Florecindiacetate (FDA)

The living nematodes got some small parts stained. However, these parts only got a light color and were hard to see in the UV-microscope. The dead nematodes got the whole body stained and under the UV-light the color was bright blue and was easy to see in the microscope, see Figure 7.

Figure 7. Dead nematode stained by FDA.

5.2.3 NaOH- test

The dead nematodes did not react to the NaOH. However, some of the dead nematodes did not have straight bodies. Living nematodes started to move and the movement became twitching and then after a couple of minutes they stopped and became straight. The sample which contained both living and dead nematodes was hard to analyze due to that there were no real difference in shape between living and dead nematodes.

6 Discussion

6.1 Extraction methods

This study indicates that the Baermann funnel extraction is the most suitable method for extracting *A. besseyi* and *D. dipsaci* from *Brachiaria* seeds. The Baermann funnel extraction was the method where most nematodes were extracted, and in addition the method was easy to execute and gave clean samples that were easy to analyze. That the Baermann funnel method extracted the most nematodes shows that the other methods are not as reliable and might not be able to extract all living nematodes after seed treatment.

The Coolen and D'Herde method was the method used by a Brazilian laboratory to determine the infection rate of the used seeds (60 nematodes/ 5 g seed). When comparing the results from testing the Coolen and D'Herde method in the present study with the infection rate, the number of found nematodes is not that different. If the infection rate of the seeds is compared to the Zuckermann et al method, it is also quite similar to the 60 nematodes/ 5 gram seed with the mean value 50 nematodes/ 5 gram seed. However the Baermann funnel method gave much higher number of recovered nematodes compared to the determined infection rate. This indicates that the Coolen and D'Herde method is not the best to use when extracting nematodes from *Brachiara* seeds.

One of the problems with the Baermann funnel method and Zuckermann et al method is that nematodes risk dying if they are in the funnels for too long without change of water or addition of new water. At the end of the test period, the number of dead nematodes in the samples slightly increased. This is likely due to lack of oxygen in the water and not because of dead nematodes being extracted from the seeds. According to van Bezooijen (2006), it is common for nematodes to die due to lack of oxygen and accumulation of microorganisms in the bottom of the funnels. Other studies have also reported this problem (Philis & Braasch, 1996; Bar-

rière & Félix, 2006). To prevent lack of oxygen, van Bezooijen (2006) recommend to use a 0.15 % solution of hydrogen peroxide instead of water and to prevent bacterial growth it is recommended to put a few ml of methyl-p-hydrobenzoate in the funnel water. How well this recommendations works is not known. It would be very interesting to test them on especially the Zuckermann et al method, because with that method there were problems with very cloudy samples.

Since *A. besseyi* often is located inside the seed compared to *D. dipsaci*, which often occur on the outside of the seeds, crushing of the seeds (in the Zuckermann method) seems like a good way of reducing the time for nematodes to extract. However, the presoaking and crushing of the seeds that should shorten the extraction time, made the samples very cloudy and hard to analyze. To get rid of the cloudiness, the samples could be passed through an even smaller mesh which catch the nematodes but let the particles pass or use one of the recommendations by van Bezooijen (2006) presented above. In the present study, the Zuckermann method already had a smaller mesh size than the Baermann funnel method. The sieves in the Zuckermann method rely on that the nematodes go straight through the mesh to pass through. This can make it more difficult for the nematodes to move from the seeds to the funnel and could be one reason why lower number were extracted than with the Baermann funnels. Also the crushing of the seeds could kill or harm the nematodes affecting their ability to pass the sieve. Coolen and D'Herde (1972) noted in their study that there are a risk of damaging and killing the nematodes if the plant samples are crushed too much. As mentioned above, in the Baermann funnel section it is a known risk that leaving the samples without changing the water or waiting too long to analyze the nematodes might die from lack of oxygen. However with Zuckermann et al method, there were dead nematodes already in the early analyses when there should not have been a problem with low oxygen amount compared to the other methods. The Zuckermann et al method had a slightly higher number of dead nematodes in the untreated seed samples. The reason to this could be that more materials from the seeds goes into the funnel due to the crushing. This material will then start to decompose which creates a lack of oxygen in the water.

A reason to the low recovery of nematodes with the Coolen and D'Herde method could be that the seeds were not presoaked. From the funnel methods, it is clear that the nematodes needs time to come out from the seeds. Presoaking the seeds gives the advantage of the nematodes coming out of the seeds by themselves whereas only crushing the seeds and not presoaking them gives the problem of finding a sufficient way of crushing. If the seeds are not crushed sufficiently, the nematodes will be left in the seeds but if the seeds are crushed too much there is a

risk of damaging the nematodes. However from the tests of different pre-soaking and crushing strategies, no differences could be seen but more repetitions are needed to be able to confirm this. Favoreto et al. (2006) performed a study where they compared the Coolen and D'Herde method with the Baermann funnel method. They concluded that the highest recovery of nematodes was by using Coolen and D'Herde method with non-presoaked seeds. However in the study by Favoreto et al. (2006), it is not stated which Coolen and D'Herde method that was used. Coolen and D'Herde developed two different paths to use depending on the nature of the nematodes. One path is developed for swollen stages and the other path for free-living stages and eggs. Since the both *A. besseyi* and *D. dipsaci* are free-living nematodes one might think to use the latter path. The path tested in this thesis, which is also the path used in the Brazilian laboratories (recommended by Favoreto et al., 2006), are the path for the swollen stages. It can also be noted that this method have originally been developed for nematodes in roots and not in seeds.

The tests of the negative control (high intensity treatment) with the Coolen and D'Herde showed that there were nematodes in the samples, and that these nematodes were dead. This could indicate two scenarios; 1.) The samples had been contaminated and 2.) The method extracts both living and dead nematodes from the seeds. In this case, due to the low number of repetitions, the first scenario is most likely although the crushing and rinsing of the seeds could make it possible to extract dead nematodes. Even though the reason for finding nematodes in the negative control is unclear it indicates that this method might not be reliable for Incotecs purposes.

The simple method was only tested with 21 seeds and from these seeds only three nematodes were extracted. This low number of tested seeds is not sufficient enough to compare to the other tested methods. To test the same amount of seeds, there would be needed more than 500 vials with one split seed to do one repetition. In this case, more than 15000 vials would have been acquired too be able to test the same amount of seeds as the other methods. As stated in the introduction, the aim this thesis was to find an extraction method that is easy, fast, cheap, and not labor intensive. This was why no further testing of this method was done, although the method has real advantages such as clear samples and the opportunity to see how many nematodes one infected seed can contain.

As I received the tested seeds from Incotec it is difficult to determine the seed lot's representativeness and further determine the generality of the results. For these reasons, it may be appropriate to test other seed lots and seeds from other *Brachiaria* species. Although there were big differences in number of recovered

nematodes between the repetitions for the untreated seeds it is not likely due because of the seed lot. A likely reason for the variance in number of recovered nematodes are environmental factors like too little or too much water in the funnels or seed samples that stood skewed in the funnels.

6.2 Staining methods

The staining method that worked best was the potassium permanganate because it successfully stained the dead nematodes, even at low concentrations. This method has previously been tested on 12 different species of nematodes including *D. dipsaci* and others like *Heterodera schachtii* and *Trichodorus* sp. (Jatala, 1975). All the 12 tested species of nematodes were successfully stained, but the tolerance (the time before the living nematodes died) for potassium permanganate differed among the nematode species. *Ditylenchus dipsaci* should according to Jatala (1975) be able to survive for 20 hours at the concentration 0.062 %. However, when this was tested for this thesis, no nematodes were found in the samples after 20 hours. This is not something that Jatala (1975) have reported and gives explanation to. One reason that the nematodes had disappeared could be that potassium permanganate is caustic and could possibly have dissolved the nematodes.

The florecindiacetate (FDA) also worked well, but a special UV- microscope was needed to analyze the samples. This method has previously been tested with *Meloidogyne javanica* and *Caenorhabditis elegans* and worked well for both species (Bird, 1979).

The NaOH-test (Chen & Dickson, 2000) kills the living nematodes, and because they then assume the same shape as the already dead ones, this method does not work well for the purpose of this thesis. This method was tested on *Heterodera glycines* stage four juveniles. According to Chen & Dickson (2000), the living nematodes changed their body shapes from straight to curved. This was the reason the method did not work for this thesis. Neither of *A. besseyi* and *D. dipsaci* have straight bodies when dead and thus making it hard to distinguish between live and dead nematodes. Their bodies have a slight c-shape when they are dead which then could be confused with the curve-shape that the living nematodes were supposed to get.

6.3 Conclusions

I would recommend Incotec to use the Baermann funnel method for testing the effect of their seed treatment. The Baermann funnel method was the method with

highest numbers of recovered nematodes and it was easy to set up for testing. Possibly, modifications such as using hydrogen peroxide instead of water and addition of methyl-p-hydrobenzoate to prevent accumulation of microorganism could make the method even better suited for Incotec's needs.

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Appendix A

Tables with Data for the Baermann funnel method

Table A1. Number of recovered nematodes from untreated seed with the Baermann funnel method.

Baermann funnel method															
Untreated seed															
	Analysis 1			Analysis 2			Analysis 3			Analysis 4			Analysis 5		
	Living	Dead	total	Living	Dead	total	Living	Dead	total	Living	Dead	total	Living	Dead	total
Repetition 1	69	0	69	25	0	25	114	1	115	61	0	61	57	0	57
Repetition 2	129	0	129	6	0	6	0	0	0	0	0	0	0	0	0
Repetition 3	127	0	127	1	0	1	6	1	7	0	0	0	0	0	0
Repetition 4	15	0	15	9	0	9	0	2	2	0	0	0	0	0	0
Repetition 5	148	0	148	33	0	33	2	2	4	0	0	0	0	0	0
Repetition 6	57	0	57	153	0	153	129	0	129	682	0	682	126	0	126
Repetition 7	44	0	44	19	0	19	21	0	21	30	0	30	24	0	24
Repetition 8	70	0	70	92	0	92	132	0	132	181	0	181	214	0	214
Repetition 9	78	0	78	90	0	90	200	0	200	580	5	585	60	2	62
Repetition 10	56	0	56	34	0	34	304	0	304	95	6	101	180	6	186
Total	793	0	793	462	0	462	908	6	914	1629	11	1640	661	8	669

Table A2. Number of recovered nematodes from Incotec intensity seed treatment with the Baermann funnel method.

Baermann funnel method															
Inotec intensity															
		Analysis 1			Analysis 2			Analysis 3			Analysis 4			Analysis 5	
	Living	Dead	total	Living	Dead	total	Living	Dead	total	Living	Dead	total	Living	Dead	total
Repetition 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 2	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0

Table A3. Number of recovered nematodes from negative control with the Baermann funnel method.

Baermann funnel method															
Negative contol															
	Analysis 1			Analysis 2			Analysis 3			Analysis 4			Analysis 5		
	Living	Dead	total	Living	Dead	total	Living	Dead	total	Living	Dead	total	Living	Dead	total
Repetition 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix B

Tables with data for the Zuckermann et al method

Table B1. Number of recovered nematodes from untreated seed with the Zuckermann et al method.

Zuckermann et al method												
Untreated seed												
	Analysis 1			Analysis 2			Analysis 3			Analysis 4		
	Living	Dead	Total	Living	Dead	Total	Living	Dead	Total	Living	Dead	Total
Repetition 1	9	5	14	4	0	4	0	0	0	0	0	0
Repetition 2	11	22	33	0	0	0	3	0	3	1	0	1
Repetition 3	44	25	69	1	2	3	3	0	3	0	0	0
Repetition 4	8	0	8	0	4	4	3	0	3	0	0	0
Repetition 5	34	5	39	1	6	7	1	0	1	0	0	0
Repetition 6	62	0	62	11	0	11	2	0	2	2	0	2
Repetition 7	31	0	31	7	0	7	2	0	2	1	0	1
Repetition 8	18	0	18	3	0	3	1	0	1	1	5	6
Repetition 9	23	0	23	15	0	15	5	0	5	9	0	9
Repetition 10	40	0	40	29	0	29	10	0	10	31	0	31
Total	280	57	337	71	12	83	30	0	30	45	5	50

Table B2. Number of recovered nematodes from Incotec intensity seed treatment with the Zuckermann et al method.

Zuckermann et al method												
Incotec intensity												
	Analysis 1			Analysis 2			Analysis 3			Analysis 4		
	Living	Dead	Total	Living	Dead	Total	Living	Dead	Total	Living	Dead	Total
Repetition 1	0	1	1	0	0	0	0	0	0	0	0	0
Repetition 2	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 3	0	1	1	0	0	0	0	0	0	0	0	0
Repetition 4	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 5	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 6	0	0	0	0	0	0	1	0	1	0	0	0
Repetition 7	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 8	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 9	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 10	0	0	0	0	0	0	0	0	0	0	1	1
Total	0	2	2	0	0	0	1	0	1	0	1	1

Table B3. Number of recovered nematodes from negative control treatment with the Zuckermann et al method.

Zuckermann et al method												
Negative contol												
	Analysis 1			Analysis 2			Analysis 3			Analysis 4		
	Living	Dead	Total	Living	Dead	Total	Living	Dead	Total	Living	Dead	Total
Repetition 1	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 2	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 3	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 4	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 5	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 6	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 7	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 8	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 9	0	0	0	0	0	0	0	0	0	0	0	0
Repetition 10	0	0	0	0	0	0	0	0	0	0	0	0
Total	0	0	0	0	0	0	0	0	0	0	0	0

Appendix C

Table C1. Numbers of recovered nematodes with the Coolen and D'Herde method.

Coolen and D'Herde

	A Untreated presoaked crushed			B Untreated presoaked whole			C Untreated whole			Q Positive control		
	Living	Dead	Total	Living	Dead	Total	Living	Dead	Total	Living	Dead	Total
Repetition 1	23	27	50	44	1	45	18	0	18	0	1	1
Repetition 2	17	47	64	47	5	52	23	0	23	0	2	2
Repetition 3	49	5	54	39	3	42	70	0	70	0	1	1
Total	89	79	168	130	9	139	111	0	111	0	4	4