Rhizobium leguminosarum and *Meloidogyne enterolobii* interactive effects on growth and nodulation of retained cowpea varieties

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DECLARATION

I, Lerato Tshiane Kgotse, hereby declare that this is my original research work and that it has never been submitted before by anyone for any degree or examination at any university other than my current submission to the University of Mpumalanga. The use of information and materials from any other sources has been fully acknowledged.

Lerato Tshiane Kgotse	25/03/2024 Date
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Prof. D. De Waele	Date

DEDICATION

To my supportive and beloved parents, Kgapyane Dunus and Nelly Madire Kgotse, and siblings, Tebogo, Thapelo, and Astro Kgotse. My nephews, Kekeletso and Thathego Modupi, and most importantly unborn child Mpho.

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ABSTRACT

Reports on the interactions of nematodes and nitrogen-fixing bacteria have been inconsistent, making nematode management decisions hard to make. A study was conducted at the University of Mpumalanga under greenhouse conditions to determine the relationship between the two rhizosphere-habiting organisms on the development of cowpea farmer retained varieties. The results obtained will clarify the relationship between the two species and develop a better management plan for nematodes, especially for the communal farmers. Interaction between root-knot nematode, *Meloidogyne enterolobii* and rhizobium, *Rhizobium leguminosarum* on six retained cowpea varieties, Cv17A, Cv17I, Cv17C, Cv17B, Cv17D and Cv17F, were evaluated in pot experiments under greenhouse conditions over two seasons, winter and summer, 2021. A 3 x 6 factorial experiment was laid out in a randomized complete block design (RCBD) with 5 replications (n = 90). The first factor consisted of the time of organism inoculation with levels of (i) *R. leguminosarum* applied a week before *M. enterolobii*, (ii) *M. enterolobii* applied a week before *R. leguminosarum* inoculation, and (iii) both microorganisms applied at the same time, whereas the second factor consisted of six farmer-retained cowpea varieties. Plastic pots of 25 cm diameter were filled with a mixture of pasteurized (250 ºC for 4hr) sandy and sandy-loam soils at a ratio of 3:1 (v/v). The pots were placed on greenhouse benches at an intra- and inter-row spacing of 0.6 and 0.5 m, respectively. Four seeds were sown in each pot and irrigated with 250 ml of tap water after every 48 hours. The seedlings were then thinned at a two-true-leaf stage to leave one seedling per pot. A week after thinning seedlings, the treatments stated above were applied. When required the inoculum of 5 000 *M. enterolobii* second-stage juveniles and (1×10^{10} CFU/ml) of *R. leguminosarum* were inoculated on the cowpea seeds depending on the order described above. At 75 days after initiation of the experimental treatments, data on plant growth, number of nematodes,

and rhizobia variables were collected. *Meloidogyne enterolobii* was able to reproduce in all six varieties as indicated by a reproductive potential of greater than one, making all the farmer retained cowpea varieties highly susceptible to the nematodes. Season had the greatest effect on varieties response to time of inoculation. Both the growth and nodulation of cowpea varieties and the population densities of nematodes infecting the plants differed greatly with season, with summer generally improving varietal growth, nodulation, and *M. enterolobii* populations and reproduction. However, the interaction of *M. enterolobii* and *R. leguminosarum* differed with each cowpea variety, Cv17A had higher numbers of active nodules when it was inoculated with *R. leguminosarum* first than the other two inoculations whereas, the inoculation time had no effect on the number of active nodules of all other varieties. In conclusion, the relationship between the nematode *M. enterolobii* and *R. leguminosarum* is much complex than previously assumed. The environment, time of inoculation and cultivar all seem to influence the interaction.'

PEER-REVIEWED PRESENTATIONS FROM THE DISSERTATION

1. Poster Conference presentations

i. **Kgotse, L.T**., Timana, M., Mnyambo, M.N., Khosa, M.C., Mbatyoti, O.A., De Waele, D., and Dube, Z.P. 2021. *Rhizobium leguminosarum* and *Meloidogyne enterolobii* effects on growth and nodulation of retained cowpea varieties. Page, 23, 23rd Symposium of the Nematological Society of Southern Africa. 2021, 19-23 September, Waterval Country Lodge, Tulbagh.

2. Oral Conference presentation

i. **Kgotse, L.T**., Timana, M., Mnyambo, Khosa, M.C., Mbatyoti, O.A., De Waele, D., and Dube, Z.P. 2021. *Rhizobium leguminosarum* and *Meloidogyne enterolobii* effects on growth and nodulation of retained cowpea varieties. ARC-TSC Postgraduate Research Open Day. 2-4 November 2021, ARC-TSC Recreation Hall.

CHAPTER 1

RESEARCH PROBLEM

1.1 Background

Cowpeas, *Vigna unguiculata* (L. Walp.), are the most economically important legume crop indigenous to Africa (Alahira, 2016). Internationally, cowpeas are produced in 12.5 million ha of land with a total grain production of 3 million tons (Alahira, 2016). In African countries, cowpeas have various uses that include livestock feeds, human consumption, and improvement of soil fertility (Alahira, 2016). When cowpea is utilized in crop rotation systems, it provides a build-up of organic matter nitrogen and carbon fixation, which results in improved soil fertility and enhanced soil physical characteristics (Sánchez-Navarro, Zornoza, Faz & Fernandez, 2019a).

Cowpea production and growth are however limited by a variety of factors, such as, severe weather conditions which are primarily a result of changing climate (Farroq, Gogoi, Barthakur, Baroowa, Bharadwaj & Alghamdi, 2017), diseases that include those caused by fungal, viral, bacterial, and nematode pathogens (Sikora, Coyne, Hallman & Timper, 2018; Gheysen & Mitchum, 2011). Worldwide plant parasitic nematodes have been overlooked and neglected by farmers because of limited knowledge of the amount of damage they cause. Nematodes have been reported to reduce crop production worth billions of dollars globally annually (Sikora, *et al*., 2018). Root-knot nematodes (*Meloidogyne* spp.) are the most important of all plant parasitic nematodes in the agricultural sector (Huynh, Matthews, Ehlers, Lucas, Santos & Ndeve, 2015). Nematode damage on yield has been reported to be at US$157 billion globally (Singh, 2015). In commercial settings the damage has been reduced with the use of synthetic chemicals, however, communal farmers have not had that privilege (Bukar, 2012). With cowpeas mainly being produced by communal

farmers with limited capital to invest in chemical control, environmentally friendly and compatible farming methods need to be recommended to the farmers (Asiwe, 2009).

In semi-arid areas, nitrogen deficiency has been identified as limiting production of cowpea, with the problem addressed by the addition of inorganic nitrogen fertilizer (Kwena, Karuku, Avuke & Esilaba, 2019). However, with the current high costs of inorganic fertilizers, the product has remained out of reach for most communal farmers where most cowpea production takes place. The cost-effective and environmentally friendly alternative to inorganic fertilizers in cowpea has been the use of nitrogen-fixing bacteria (Abdel-Fattah, Rabie, Lamis & Rabab, 2016). A biological nitrogen fixation is the outcome that is produced from the relationship between cowpea and rhizobia, which is of benefit to the plant as its nitrogen needs are met (Ngakou, Nwaga, Ntonifor, Tamo, Nebane & Parh, 2007).

1.2 Justification

The nitrogen-fixing bacteria and leguminous plant relationship is affected by other soil organisms, such as root-knot nematodes (Soares, Trejo, Veloso & Videira, 2016). Contradictions on the impacts of the interaction between root-knot nematodes and bacteria have been reported several times (Soares *et al*., 2016; Costa, Ng & Mathesius, 2021). Some scholars report that root-knot nematodes damage the plant roots and reduce nodulation, whereas other reports indicate that the presence of nitrogen-fixing bacteria reduces the severity of root-knot nematodes (Khan, Anwer, Khan & Haque, 2012; Costa *et al*., 2021).

Khan *et al*. (2012) reported that cysts and root-knot nematodes are the cause of reduced nodulation in legume plants. The study observed nematode species, *Heterodera trifollli* inhibition of nodules

on soybean and *Meloidogyne* spp. on peanuts completely inhibiting nodulation (Khan *et al*., 2012). Masefield in a study conducted in 1958 stated that the reason for reduced nodulation in legume crops is due to the indirect effect from nematode galls which causes damage to the root system of the plant and also competition for space in the rhizosphere is a contributing factor (Khan *et al*., 2012). Khan *et al.,* (2012) reported that the *M. incognita-Rhizobium japonicum* relationship results in competition between the two species as they occupy the same space in the rhizosphere, this could therefore mean the organism that colonizes the rhizosphere first could be the one dominating it. Khan *et al*., (2018) later reported that the relationship between the two can be more than competition for rhizosphere space but the ability of nematode to infect even the bacterial nodules. Neeraj and Singh, (2019) in their study observed a reduction in the efficacy of *Rhizobium leguminosarum* in the presence of *Meloidogyne* species. In contrast, Veken *et al*., (2020) reported a reduction in the impact of nematodes on plants inoculated with rhizobacteria. These contradictions in the relationship between these organisms deprive farmers of a potential economic and environmentally friendly method of improved cowpea production.

The aim of this study is to examine the correlation between *Meloidogyne enterolobii*, a newly discovered root-knot nematode that can have significant economic impact on soybeans, and *Rhizobium leguminosarum* in terms of its impact on the growth of local cowpea varieties. This study aims to explore the potential use of Rhizobium leguminosarum in the management of nematodes in communal farming areas.

1.3 Problem statement

The reports on the interaction of nematodes with nitrogen-fixing bacteria have been inconsistent making nematode management decisions hard to make (Veken, Win, Seeboruth, Cabasan,

Swennen, Elsen *et al.,* 2020). Hallman, Quart-Hallman, Mhaffe & Kloeper, (1997) observed a reduced *M. incognita* disease severity when nitrogen-fixing bacteria *Rhizobium leguminosarum* is present and explained that the reason was due to *Rhizobium leguminosarum* out competing nematodes for nutrients. Some scholars report that the interaction was more than just competition for nutrients that lead to nematode disease suppression, but unknown stress factors played a key role in the interaction (Veken *et al*., 2020). There are also reports that nematode invasion of the roots directly or indirectly results in turning active nodules into inactive nodules (Costa *et al*., 2021). Nematode infection can affect the content of leghemoglobin in nodules reducing the activity of nitrogenase (Khan *et al*., 2018). The current study attempts to contribute to the knowledge through the determination of the relationship between the *Meloidogyne enterolobii* and *Rhizobium leguminosarum* in retained cowpea production.

1.4 Purpose of the study

1.4.1 Aim

Determination of the interactive effects of *Rhizobium leguminosarum* and *Meloidogyne enterolobii* on the growth of six farmer-retained cowpea varieties and severity of nematode disease.

1.4.2 Objective

To determine whether *Rhizobium leguminosarum* will reduce the severity of *Meloidogyne enterolobii* and improve the nodulation and growth of six farmer-retained cowpea varieties.

1.4.3 Hypothesis

Rhizobium leguminosarum reduces the severity of *Meloidogyne enterolobii* on six farmer-retained cowpea varieties and improves the nodulation and growth of six farmer-retained cowpea varieties.

1.5. Reliability, validity & objectivity

When a variable is being measured repeatedly without it changing and the measuring instrument used produces results that are consistent, it is then described as reliability (Leedy & Ormord, 2005). Numerous reliabilities checks on data are provided by statistical analyses (Berenson & Levine, 1996). In this study, appropriate levels of significance for mean separation were used to ensure reliability in numerous experiments performed and when the evaluation of variance is described by models as measured by a coefficient of determination (R^2). Validity is explained as the extent to which a measuring instrument measures what it was meant to measure (Leedy & Ormord, 2005). In empirical research, an increase in the range validity from which conclusions are drawn is done by replicating experiments in time or space. Performing experiments in the same location after some time ensures validity (Little & Hills, 1981). Relying on verifiable data by striving as far as possible to get rid of biases or subjective evaluations is termed objectivity (Leedy & Ormord2005). The discussion of results on basis of empirical evidence is displayed by statistical analysis, with the comparison and contrasting of results with other results obtained from other studies (Little & Hills, 1981).

1.6. Bias

Any set of conditions or influences that distort data whether it is done independently or together is termed as bias (Leedy & Ormord, 2005). In this study, bias was reduced by checking that error in each experiment were minimized through replication and randomization (as followed in Little & Hills, 1981).

1.7. Scientific contribution of the study

Rampant contradictions in the literature on the relationship between nitrogen-fixing bacteria and root-knot nematodes create a challenge in management decisions targeted at environment-friendly nematode management. The current study would add to empirical evidence that attempts to provide clarity and more insight into the relationship between the two organisms. The findings would provide valuable and important information to communal farmers on the opportunities available for the management of *M. enterolobii*.

1.8 Structure of dissertation

Chapter 1 covers a detailed description of the research problem and Chapter 2 covers information on the work not done and work done in this project. Chapter 3 describes the findings of the objective of the study, and the last chapter, Chapter 5, wraps up the document with a summary, the significance of findings, future research, and conclusions. Each chapter is a stand-alone with its references. The referencing style used in the document is Harvard style as approved by the Senate of the University of Mpumalanga.

1.9 References

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CHAPTER 2

LITERATURE REVIEW

2.1 Cowpea production in South Africa

Cowpeas, *Vigna unguiculata* (L.) Walp is known for its tolerance to drought and low soil fertility. The level of production of cowpeas in South Africa is not as high as other crops, such as maize, which is economically important (National Department of Agriculture, 2014). The producers of cowpea are small-scale farmers who cultivate the cowpea crop under dryland conditions. The size of the land where the crop is produced, and the quantity of the crop produced by the small scale-producers in South Africa is not known nor has it been recorded to date (National Department of Agriculture, 2014). The reason behind this is that cowpeas are cultivated as intercrops with other vegetables.

The production and research of cowpeas in South Africa have been undervalued by researchers for the last 30 years due to the absence of funding from the government and researchers having less interest in improving the crop (Asiwe, 2007). With the lack of funding and interest in research for cowpea improvement, cowpea varieties remain unimproved which leads to the planting of seeds of poor quality, and this results in low yield productions. Hence in South Africa, the production of the cowpea crop is at a subsistence level with a lot of work that needs to be done by scientists (Asiwe, 2009). The production areas of cowpeas in South Africa are Limpopo, Mpumalanga, and KwaZulu-Natal (Asiwe, 2009), Rainfall is between 750–1000mm in some of the regions in the Mpumalanga province (Asiwe,2009).

2.2 Economic importance of cowpea

Cowpeas are generally cultivated for their edible seeds, pods, and leaves for human consumption. It is mostly cultivated by small-scale farmers who also cultivate it to feed their livestock, cowpea provides forage, hay, and silage. Cowpeas are also used as a natural fertilizer and cover crops for the maintenance of the soils (Alemu, Asfaw, Woldu, Fenta & Medvecky, 2016). It has a positive impact on soils by compensating for the loss of nitrogen which is taken up by cereals because of their ability to grow in poor soils and fix atmospheric nitrogen, with all its abilities it is considered a promising crop in tropical areas (Alemu *et al*., 2016; Belay, Gebreslasie & Meresa, 2017; Bilatu, Binyam, Solomon, Eskinder & Ferede, 2012). The estimated land used to produce cowpeas worldwide every year is estimated to be 14.5 million ha with a yearly production of 6.2 million metric tons (FAOSTAT, 2016). The world's production area for cowpeas is 84% of land area with 83.4 % overall production from Africa. Of which 80 % of the 83.4% of the production is from West Africa (FAOSTAT, 2016).

Cowpeas also serve as a source of income in many African countries, small-scale farmers usually sell and trade leaves and grains within their communities (Alemu *et al*., 2016). Cowpeas are traded in all the local markets in countries such as Nigeria, Mali and Senegal. Farmers benefit from cowpeas because the crop is a cash-generating commodity. Both urban and rural communities benefit from the trading of cowpea fresh leaves and processed food, especially for women, because it provides opportunities to earn money that will assist in maintaining their livelihoods (Ngalamu, Odra & Tongun, 2015).

2.3 Factors affecting cowpea production

The low production of cowpeas is mainly caused by a variety of biotic and abiotic factors. The utilization of cowpeas as intercrops with cereals in or on land that partially supports agricultural activities leads to the competition for moisture and nutrients as the cereals will grow taller than the cowpeas making the environment unfavorable to the growth of the cowpeas. Most cowpeas grown by smallholder farmers do not use fertilizers and plant protection measures to promote the growth of the plant thus the damage caused by pest insects causes severe yield losses (Ajeigbe, Singh, Adeosun & Ezeaku, 2010). In Southern Africa, another factor contributing to low yield is the lack of improved cowpea varieties that can produce well under environmental stresses and poor production practices (Ajeigbe *et al.*, 2010).

2.3.1 Biotic factors

Fungal diseases: Several fungal species cause disease in cowpea, for example, the leaf smut also known as false smut which is caused by *Protomycopsis phaseoli* (Bailey, Nash, O'Connell & Skipp, 1990). Root rot, stem rot, and leave smut in cowpeas are caused by fungal diseases, In Nigeria, Sudan Savanna there have been reports of yield loss due to serious epidemics where 100 % loss occurs in most areas (Mbeyagala, Mukasa, Tukamuhabwa & Bisikwa, 2014).

Bacterial diseases: Bacterial diseases affect almost every part of the plant (Viswanatha *et al*., 2011). Bacterial diseases cause reductions in yield where up to 71 % in pod loss, 68 %, and 53 % in seed and fodder loss, respectively, especially in susceptible varieties. The bacterial blight which is caused by *Xanthomonas campestris* pv. *vignico* is a well-known and serious bacterial disease (Viswanatha *et al*., 2011). Symptoms caused by bacterial blight during moderate infection include yellowing of the leaves and the presence of irregular round spots resulting in drooping of leaves (Viswanatha *et al*., 2011).

Viral diseases: More than 20 viruses affect the production of cowpea globally, the identified viruses cause yield losses of up to 90–100 % (Mbeyagala *et al*., 2014). Aphids are vectors of viral diseases that are said to cause up to 100 % crop losses in cowpea production (Horn *et al*., 2015). Some of the cowpea viral diseases affect root nodulation (Taiwo et al., 2014). The red mosaic virus negatively affects the growth and development of *Rhizobium* bacteria leading up to 20–45 % of nodulation reduction (Taiwo *et al*., 2014).

Insect pests: Insect pests affect crops that are stored and those in the field. The insect pests of cowpea include bruchids, beetles (*Ootheca mutabilis*), leaf hoppers, and foliage beetles (Ngakou Nwaga, Ntonifor, Tamo, Nebane & Parch, 2007). They are said to be present throughout the vegetative growth stages acting as virus vectors and when they feed on the leaves of the plant (Ngakou *et al*., 2007). In Namibia, bruchids, a post-harvest pest that attacks stored grains, causes up to 100 % yield losses, (Horn *et al*., 2015).

Root-knot nematodes: Root-knot nematodes cause severe losses in the agricultural industry, especially in crop production. *Meloidogyne incognita* and *Meloidogyne javanica* are the well-known root-knot nematode species that reduce production in cowpea. These nematodes cause damage by interfering with the uptake of nutrients and water and the transportation of auxin, the differentiation of plant cells becomes limited in the roots of the plant, and they also make the host plants vulnerable to infection by soil-borne pathogens (Gheysen & Mitchum, 2011; Haegeman *et al*., 2012).

2.3.2 Abiotic factors

During the start and towards the end of the rainy season cowpeas suffer from the effects of erratic rainfall which lead to yield losses (Daryanto, Wang, & Jacinthem, 2015). During terminal drought varieties that mature early can survive and produce good yields but when there is irregular moisture stress, especially during the vulnerable vegetative stages, they won't produce good yields (Hall, 2004). Cowpeas are known as crops that tolerate drought more when compared to other crops but continuous exposure to drought is detrimental, this mostly occurs in areas where there is rainfall scarcity and irregular rainfall patterns like in the regions of Sub-Southern Africa (Boukar, Fatokun, Huynh, Roberts & Close, 2016).

Drought: Drought is a major factor affecting the production of cowpeas. One of the cowpea's traits is drought resistance but it can negatively be affected by severe drought during the stages of pod setting and grain filling (Hall, 2004). When drought stress occurs during the flowering stage the grain yield falls from 1000 kg. ha^{-1} to 360 kg. ha^{-1} (Boukar *et al*., 2016).

Socio-economic factors: Horn *et al*. (2015) have outlined socio-economic factors that affect cowpea production in Sub-Sahara Africa, those factors include low yield potential, lack of improved varieties, the costs of land preparation, high cost of pesticides, poor harvest prices, lack of proper harvesting tools and marketing channels. In Nigeria, cowpeas are produced on large scales but there are economically important socio-economic factors such as the unavailability of an established value chain and less development of cowpea as a commodity crop (Aboki & Yuguda, 2013).

2.4 Factors affecting nitrogen-fixing bacteria performance.

2.4.1 Benefits of rhizobia

Rhizobia are found in the soils, and they play an important role in plants because they promote plant growth. Rhizobia occupies the plant rhizosphere where they obtain nutrients from the root exudates and in return, they benefit the plant by enriching the soils with nutrients such as nitrogen (N), phytohormones production, phosphate solubilization, and siderophore production (Gopalakrishnan, Sathya, Vijayabharathi, Varshney, Gowda & Krishnamurthy, 2015).

Rhizobia also increases plant protection through the interference of cellulose, lipase, protease, and β-1.3 glucanase production while inducing systematic resistance through acetoin, lipopolysaccharides, butanediol, flagella and against pests and diseases in which it enhances plant defense (Gopalakrishnan *et al*., 2015).

Indirect plant growth promoters:

Certain rhizobia species such as *R. leguminosarum bv. Tri-folli, R. leguminosarum bv. Viciae, R. meliloti, and R. trifolii*, have bio-control properties which allow them to act as plant promoters (Bardin, Huang, Pinto, Amundsen, & Erickson, 2004). According to Bardin *et al*., 2004 these properties allow rhizobia to inhibit the growth of undesired organism and pathogens. Mechanisms of the bio-control properties include nutrient competition and antibiotic production (Arora, Kang &Maheshwari , 2001). For example, *R. meliloti R. leguminosarum bv. tri-folli*, and *R. trifolii R leguminosarum bv. viciae* produce enzymes that degrade cell-wall and antibiotics that can inhibit plant pathogens (Bardin *et al*.,2004) . Some rhizobial strains can limit the growth of pathogens by producing high affinity siderophore's which starve the pathogens of the available iron (Gopalakrishnan *et al*., 2015).

Direct plant growth promoters:

Rhizobia can be used as an inoculant to enhance nitrogen fixation. Studies done on different strains of rhizobia confirmed that rhizobia are effective soil colonizers that can persist in the soil even without the host plants (Gopalakrishnan *et al*., 2015). The symbiosis between rhizobia and legumes is reported to be a much cheaper source of nitrogen and it is an agronomic practice that is effective when it comes to checking that there is an adequate supply of nitrogen compared to nitrogen fertilizer application (Gopalakrishnan *et al*., 2015).

2.4.2 Nitrogen fixing bacterial constraints.

Soil stress: Salinity in the soil is caused by poor soil drainage, irregular irrigation, and incorrect fertilizer application (Adil, Kant & Turan, 2012). Most legumes that depend on nitrogen fixation require soils that are slightly acidic and neutral for growth (Zahran, 1999). The host plants have higher sensitivity than their rhizobia counterparts (Zahran, 1999). The development and metabolism of nodules and the processes of the symbiotic interactions are affected by salt stress, this results in the development of a low number of nodules (Ogutcu, Kasimoglu & Elkoca, 2010). High concentrations of salt in the soil causes direct toxicity and affects microbial populations present in the soil. The multiplication of *Rhizobium* spp. is affected by soil salinity leading to the prevention of infection taking place which then directly affects root nodule functions, plant growth, and nitrogen demand (Ogutcu, *et al*., 2010). The response of rhizobia to salt stress involves processes that are physiological and biochemical and affect the colonization of rhizobia on the roots of the plants (Nabizadeh, Jalilnejad & Armakani, 2011).

Temperature:

High temperatures between 34 and 47°C are more detrimental than low temperatures when it comes to soil rhizobia survival because they affect the processes of bacteroid performance and functioning of nodules and the root hair infection, nodule structure, bacteroid differentiation, and the functioning of the legume root nodule (Zahran, 1999). Temperatures for growth vary between strains and species. The activities of rhizobia are altered by temperature, for example, if a certain rhizobia species can be effective at a certain temperature, then it won't be effective at other different temperatures. Multiplication of rhizobia in the soil takes place at temperatures of up to 42 °C.

Drought: Rhizobia can survive under soil conditions that have limiting moisture levels, the growth and multiplication of rhizobia and the symbiosis process of rhizobia are affected by drought conditions (Zahran, 1999). Legume plant productivity is affected by drought because drought causes osmotic stress and viable strains of *Rhizobium* are unable to function under such stress (Zahran, 1999).

Soil moisture: Nodule initiation and growth are sensitive to moisture, therefore, lack of soil moisture inhibits the development of nodules (Manoj, Kaila, Singh, Gangola & Dhawan, 2011). The development of nodules is affected more directly by water stress at vegetative and tasseling plant growth stages than any other stage in which the recovery of the nodule development is highly impossible (Manoj *et al.,* 2011).

2.5 Interactions of nematodes with other pathogens

2.5.1 Nematode-fungus wilt disease interaction

Many studies have reported that nematodes have included a fungus as their component. The interrelationship involves a nematode pathogen and one wilt-inducing fungus. Studies have reported that in a cotton plant, the severity of the *Fusarium wilt* increased with the presence of root-knot nematodes (*Meloidogyne spp*) (Zuckeman, 2015). Given the interaction between these two pathogens, a lot of attention was given to different host plants (Zuckeman, 2015). A study was conducted on tomato plants (Solanum lycopersicum) to examine the relationship between Fusarium and root-knot nematodes. The findings indicated that nematode infection allowed certain strains of F. oxysporum f. lycopersici to attack tomato varieties that were initially resistant to those strains. It was concluded that the fungus mutates within the host plant (Zukerman, 2015).

2.5.2 Nematode- fungus seedling disease

The interrelationship between nematode and fungi causes severe damage to crops due to disorders that are classified as seedling diseases. Citrus nematodes under greenhouse conditions interact with *F. solani* and cause a reduction in the growth of citrus seedlings (Zuckeman, 2015). The effect of two pathogens on plants causes greater damage than one pathogen (Zuckeman, 2015).

2.5.3 Nematode- bacteria wilt interactions

Bacterial wilt, *Pseudomonas solanacearum,* causes severe damage when nematodes are present in the host plant (Zuckeman, 2015). In tomato plants, the presence of root-knot nematode, *M. hapla,* and Spiral nematode, *Helicotylenchus nannus,* cause an increase in the development of wilt, and in tobacco, *M. incognita* has a great influence on the development of bacterial wilt. In a study that was conducted on host plants of *P. solanacearum*, the host plants were exposed to nematodes 4

weeks before being exposed to *P. solanacearum* and the results showed that the host had severe wilt symptoms earlier than when the host was exposed to the bacteria first (Zuckeman, 2015). Plant varieties that were resistant to nematodes did not develop any wilting symptoms when exposed to both pathogens (Zuckeman, 2015).

2.5.4 Nematode-virus interactions

In plant pathology, the virus-nematode vector relationship is a very significant aspect (Zuckeman, 2015). The growth of root-knot nematode rapidly increases in the roots of susceptible tomato plants when infected with tobacco mosaic virus than in roots of plants that are virus-free. In some cases, there are no observable effects of the virus on the number of nematodes that can enter the roots. The preliminary research that was conducted in North Carolina reported that certain viruses and nematodes interact within the host plant (Zuckeman, 2015). Root-knot nematodes, such as *Longidorus* sp. transmit plant viruses through consuming the virus from one crop and transmitting it to the next crop (Zuckeman, 2015).

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CHAPTER 3

EFFECTS OF *RHIZOBIUM LEGUMINOSARUM* ON SEVERITY OF *MELOIDOGYNE ENTEROLOBII* AND GROWTH OF SIX FARMER- RETAINED COWPEA VARIETIES

3.1 Introduction

Root-knot nematodes are known to be part of the most important fauna affecting economic crop production. It is important to find cost-effective and environment-friendly strategies for controlling nematodes. For example, controlling nematodes chemically has been effective but has affected the environment and human health negatively (Mostafa *et al*., 2014). Biological control of nematodes is explained as a way of reducing the nematode population and damage through the action of other organisms that are antagonistic to them, some of which can be introduced into the environment. The introduced organism can interact directly with the pathogen which can be through antibiosis and competition for nutrients and space or interact indirectly by inducing plant resistance (Xiang, Lawrence & Donald, 2018). Plant growth-promoting bacteria such as rhizobacteria can colonize the tissues of living plants. The bacteria enhance plant growth and reduce damage caused by soil-borne plant pathogens, interestingly some of the plant growth-promoting rhizobacteria show nematicidal activity (Poveda, Abril-Urias & Escobar, 2020). *Pseudomonas fluorescens* is reported to produce secondary metabolites that can cause mortality of nematode eggs and second stage infective juveniles (Poveda *et al*., 2020). Therefore, the objective of this study was to determine whether *Rhizobium leguminosarum* will reduce the severity of *Meloidogyne enterolobii* and improve the growth and nodulation of six farmer-retained cowpea varieties.

3.2 Methods and materials

3.2.1 Study Location

The study was conducted at the University of Mpumalanga (25º26’11’’S, 30º58’54’’E) under controlled greenhouse conditions during the summer (September, October, November, December) and repeated in the winter (May, June and July) of 2021.

3.2.2 Experimental design

A 3 x 6 factorial experiment was laid out in a randomized complete block design (RCBD) with five replications (n = 90). The first factor consisted of the time of organism inoculation with the following levels of (i) *R. leguminosarum* applied a week before *M. enterolobii*, (ii) *M. enterolobii* applied a week before *R. leguminosarum* inoculation and (iii) both microorganisms applied at the same time, And the second factor consisted of six farmer-retained cowpea varieties, Cv17A, Cv17I, Cv17C, Cv17B, Cv17D and Cv17F.

Figure 3.1 Cowpea plants inoculated with *Rhizobium leguminosarum* and *Meloidogyne enterolobii*

3.2.3 Procedure

This experiment was established in plastic pots of 25 cm diameter filled with a mixture of pasteurized (250 ºC for 4hr) sandy and sandy loam soils in a ratio of 3:1 by volume (v/v) (Figure 3.1). The pots were placed on greenhouse benches at an intra- and inter-row spacing of 0.6 and 0.5 m, respectively. Four seeds were sown in each pot and irrigated with 250 ml of tap water after every 48 hours. The seedlings were then thinned at a two-true-leaf stage to leave one seedling per pot. A week after thinning seedlings, treatments were applied as stated above. When required the inoculum of 5 000 *M. enterolobii* second-stage juveniles and (1×10^{10} cfu/ml) of *R. leguminosarum* were then inoculated to seedlings depending on the treatment.

3.2.4 Nematode inoculation

The *M. enterolobii* population to be used in this experiment was obtained from the roots of a susceptible tomato cv. 'Floradade' grown under greenhouse conditions. Inoculation was done by carefully dispensing the nematodes on the cardinal point of the plant using a plastic syringe.

3.2.5 Rhizobium inoculation

Rhizobium leguminosarum strain-23 was obtained from Mooiplaats products (UPL South Africa (Pty) Ltd, Pretoria) and used as stated in 3.2.3. The inoculation was done by coating the cowpea seeds with *R. leguminosarum* before planting. For the level that requires the inoculation of *R. leguminosarum* after nematode inoculation, the rhizobium was dispensed on the cardinal point of the plant using a plastic syringe.

3.2.6 Data collection

At 75 days after initiation of the experimental treatments, data on plant growth, nematode, and rhizobia variables were collected.

Plant variables: Plant height was measured from the soil level to the tip of the plant's flag leaf. The number of leaves was determined by counting all fully matured true leaves per plant. The shoots were then cut, and oven dried at 55 ºC for 3 days in an incubator (Model 30-1060, Quincy Lab Inc., Chicago) and weighed to obtain dry shoot mass. The root system of plants was removed from each pot and immersed in water to remove the soil particles, blotted dry with a paper towel, and weighed. Stem diameter was measured using a Vernier caliper (GV9370, Grip, Johannesburg) 5 cm from the distal cut stem end of each plant. Pod numbers were determined by counting the number of all mature pods per plant, the pods were then weighed to determine the pod mass using a laboratory scale (LABOTEC, Model: YP20002B, China).

Nematode variables: The number of root galls were assessed using a North Carolina scoring system (Table 3.1) (Taylor & Sasser, 1978). The eggs and second-stage juveniles (J2) of nematodes were extracted from the total root system/ plant by maceration and blending for 30 s in 1 % NaOCl solution (Hussey and Barker, 1973). The material was then passed through a nested 150 µm, 45 µm, and 25 µm mesh sieves, with the nematodes collected from the 25 µm mesh sieve (Jenkins, 1964). The nematode eggs and J2 in soil were extracted using the sugar-floatation and

centrifugation method (Marais *et al*., 2017). The nematode eggs and J2 from the soil and the roots, were then counted from a 1-ml aliquot placed under a stereomicroscope (Model CX23RTFS2, Olympus Corporation, Tokyo) at X40 magnification.

Table 3.1: Root galls scoring system

Description	Score
No galls	0
1-2 galls	1
3-10 galls	2
11-30 galls	3
31-100 galls	4
>100	5

Rhizobia variables: Total number of nodules, active nodules, and inactive nodules were determined. The total number of nodules were determined by counting the total nodules per root system. The presence of leghaemoglobin was used to determine whether the nodule is actively fixing the nitrogen or not, by observing the color inside the nodules. Green, brown, and white internal nodule color indicated that the nodules were inactive, whereas purple to red color meant that the nodule is actively fixing the nitrogen (Figure 3.2) (Corbin *et al*., 1977). The position of the nodules on the root system was determined using Somasegaran & Hoben, (1994) scoring system (Table 3.2).

Table 3.2: Nodule position on the root system scoring

Description	Score
• Both crown and lateral nodulation	3
• Mostly crown nodulation only	2
• Mostly lateral nodulation only	1

Figure 3.2. Active (pink) and non-active (brown) *Rhizobium leguminosarum* nodules from cowpea roots.

3.2.7 Data analysis

Data on plant, nematode, and Rhizobia variables were subjected to analysis of variance (ANOVA) through Statistix 10 software. Before the data were subjected to ANOVA, normal residual distribution was determined using the Shapiro-Wilk test, and the data that failed the normality test ($P \leq 0.05$) were transformed using Log_{10} (x+1). Mean separation was achieved through Fisher's least significant difference test at 5 % for all significant ($P \leq 0.05$) variables. The data collected were subjected to Pearson's correlation analysis to determine the relationship between independent and dependent variables, using the following formular:

$$r = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \bar{y})^2}}$$

Where:

- r = Pearson Coefficient
- n= number of pairs of the stock
- $\sum xy$ = sum of products of the paired stocks
- $\sum x$ = sum of the x scores
- $\sum y$= sum of the y scores
- $\sum x^2$ = sum of the squared x scores
- $\sum y^2$ = sum of the squared y score

Unless otherwise stated, all means were compared at a 5 % significant level.

3.3 Results

3.3.1 Interaction of experimental factors and measured variables

All data on plant, nematode, and rhizobium variables were not normally distributed ($P \leq 0.05$), hence were transformed, accordingly (Appendix 3.1 and 3.2). Factor interactions were not statistically significant ($P > 0.05$) for all measured nematode and plant variables except for Season*Variety interaction, on plant diameter, plant height, fresh root mass, dry shoot, nematode eggs in roots, total nematodes and reproductive potential (Appendix 3.3-3.6, 3.8, 3.10 and 3.11), and Season*Application time interaction, on nematode J2 in root, nematode eggs in root and nematode reproductive potential (Appendix 3.8, 3.9 and 3.11). Factor interactions were significant ($P \leq 0.05$) for all rhizobium variables. Single factor effects were not statistically significant ($P > 0.05$) for all the rhizobium, plant, and nematode variables, except for stem diameter, fresh root mass, plant height, dry shoot mass, nematode J2 in root, nematode eggs in root, nematode J2 in soil, total nematodes and the reproductive potential (Appendix 3.3-3.11).

When the mean sum of squares was partitioned, the source of variation that contributed more to total treatment variation on plant variables was Season followed by Variety, with the lowest being the interaction of Application time*Season (Table 3.3 and 3.4).

Table 3.3: Contributions of sources of variation to total treatment variation (TTV) for plant variables

Source of variance	DF	Plant height		Stem diameter		Dry shoot		Fresh root	
		MS	%	MS	%	MS	%	MS	%
Replication	4	0.213	3	0.000	0	0.016	0	0.030	1
Season (S)	1	6.963	88	0.105	83	3.179	91	2.553	73
Variety (V)	5	0.286	4	0.009	7	0.106	3	0.469	14
Application time (A)	2	0.025	0	0.003	2	0.042	1	0.055	2
S*V	5	0.307	4	0.007	6	0.066	2	0.135	4
S*A	2	0.018	0	0.000	0	0.004	0	0.030	1
V*A	10	0.044	0	0.001	1	0.025	1	0.073	2
S*V*A	10	0.034	0	0.001	1	0.026	1	0.061	2
Error		0.052	1	0.000	0	0.017	0	0.052	2
Total		7.942	100	0.126		3.481	100	3.458	

DF-degrees of freedom. MS-mean sum of squares, Nema- nematode, J2- second stage juveniles, %-percentage contribution of source of variation to TTV.

Table 3.4: Contributions of sources of variation to total treatment variation (TTV) for nematode variables

		Nema J2 roots		Nema eggs roots		Nema J2 soil		Total Nema		Reproductive potential	
Source of variance	DF	MS	%	MS	%	MS	%	MS	%	MS	%
Replication	4	1.315	1	0.872	1	2.947	2	0.403	1	0.750	0
Season (S)	1	215	94	145	88	116	91	71.046	89	138	91
Variety (V)	5	2.376	1	3.143	2	1.227	1	1.222	2	1.864	1
Application time (A)	2	1.719	1	4.186	3	1.992	2	2.045	3	2.729	2
S*V	5	1.258	1	3.053	2	1.135	1	1.412	2	1.785	1
S*A	2	3.698	2	6.494	4	0.302	0	0.847	1	4.780	3
V*A	10	1.411	1	0.905	1	1.883	1	1.069	1	1.040	1
S*V*A	10	0.737	0	0.636	0	1.017	1	0.772	1	0.191	0
Error	135	1.096	0	1.092	1	1.659	1	0.579	1	0.744	0
Total	174	228.61		165.381		128.162		79.395		151.883	

DF-degrees of freedom. MS-mean sum of squares, Nema-nematode, J2- second stage juveniles, %-percentage contribution of source of variation to TTV.

3.3.2 Effect of *Rhizobium leguminosarum* and *Meloidogyne enterolobii* application time and season on nematode variable

The differing effects of the application time of either nematode or bacteria on second-stage juveniles (J2) in root, eggs in root and reproductive potential were only observed in winter, yet in summer, the three treatments did not differ (Table 5). Generally, in winter, application of *M. enterolobii* first had significantly higher number of J2 in root, eggs in root, and reproductive potential (Table 3.5).

Table 3.5. Interaction of *Rhizobium leguminosarum* and *Meloidogyne enterolobii* application time and season on nematode variables.

	Season					
	Winter	Summer	Winter	Summer	Winter	Summer
Treatment	NematodeJ2 in root		Nematode eggs in root		Reproductive Potential	
Nematodes first	1.709[b]	3.360[a]	2.218[b]	3.374[a]	2.399[b]	3.513[a]
Both organisms	1.254[bc]	3.711[a]	1.185[c]	3.677[a]	1.654[c]	3.799[a]
Rhizobium first	0.922[c]	3.502[a]	1.341[c]	3.204[a]	1.500[c]	3.554[a]
P-value	0.0372		0.0033		0.0022	
F-value	3.37		5.95		6.42	
$LSD_{0.05}$	0.5606		0.5595		0.4544	

*J2 -Second-stage juveniles. Column means followed by the same letter are not significantly different at $P \leq 0.05$, according to Fisher's least significant difference

3.3.3 Effect of variety and season on nematode variables

Similar to 3.3.2, a profound effect of varieties was observed in winter, with summers generally, indicating no varietal differences on nematode eggs in root, total nematodes, and reproductive potential (Table 3.6). During the winter season, variety Cv17A had the highest number of nematode eggs in root, and this was not different from Cv17C (Table 3.6). The second highest was

with varieties Cv17F, Cv17I, Cv17B and Cv17D which were not different from each other (Table 3.6).

When total nematodes were considered, Cv17A had the highest yet not different from varieties, Cv17C, Cv17F, and Cv17I. Again, Cv17D had the lowest total nematodes when compared to other varieties, even though it was not different from varieties Cv17l and Cv17B (Table 3.6).

Variety, Cv17A had the highest nematode reproductive potential compared to other varieties, but was statistically similar to variety Cv17C, Cv17F, Cv17I, and Cv17B, with variety Cv17D having the lowest reproductive potential which was not different from Cv17B (Table 3.6).

Table 3.6. Interaction of variety and season on nematode variables.

	Season					
	Winter	Summer	Winter	Summer	Winter	Summer
Variety	Nema eggs roots		Total nematodes		RP	
Cv17C	2.070^{cd}	3.266^{ab}	3.212^{bc}	4.422^{a}	2.155^{c}	3.614^{ab}
Cv17A	2.360^{c}	3.489^{ab}	3.465^{b}	4.291^{a}	2.157^{c}	3.562^{ab}
Cv17F	1.585^{de}	3.728^{a}	3.570^{b}	4.467^{a}	2.089^{c}	3.983^{a}
Cv17I	1.319^{de}	2.809^{bc}	3.181^{bc}	4.249^{a}	1.940^{c}	3.133^{b}
Cv17B	1.300^{de}	3.750^{a}	2.522^{d}	4.467^{a}	1.577^{cd}	3.922^{a}
Cv17D	0.851^{e}	3.468^{ab}	2.742^{cd}	4.337^{a}	1.187^{d}	3.516^{ab}
P-value	0.0195		0.0373		0.0404	
F-value	2.80		2.44		2.40	
$LSD_{0.05}$	0.8255		0.5491		0.6619	

*Reproductive potential (RP). Column means followed by the same letter are not significantly different at $P \leq 0.05$, according to Fisher's least significant difference

3.3.4 Effect of variety and season on plant growth variables

As with nematode variables described above, plant variables were higher in summer that in winter season (Table 3.7), but unlike nematode variables, performance of varieties differed significantly in both seasons (Table 3.7).

In winter, variety Cv17A had the highest stem diameter, plant height, fresh root mass and dry shoot mass, whereas, in summer variety Cv17D, generally ranked first in all four measured variables (Table 3.7). Variety CV17I had generally the lowest of all measured significant plant variable (Table 3.7). All other variables were generally not statistically different from each other (Table 3.7)

Table 3.7: Interaction of cowpea variety and season on plant growth variables.

	Season							
Variety	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
	Stem diameter		Plant height		Fresh root mass		Dry shoot mass	
Cv17C	0.133^{g}	0.183^{b}	1.381^{g}	1.937^{b}	0.841^{bcd}	0.991^{b}	0.371^{ef}	0.605^{bc}
Cv17A	0.161^{cde}	0.172^{bcd}	1.549^{def}	1.836^{bc}	0.944^{bc}	0.995^{b}	0.430^{de}	0.619^{b}
Cv17F	0.138^{fg}	0.183^{b}	1.409^{fg}	1.644^{de}	0.721^{d}	0.991^{b}	0.312^{fg}	0.547^{bc}
Cv17I	0.102^{h}	0.157^{def}	1.466^{fg}	1.851^{bc}	0.468^{e}	0.792^{cd}	0.219^{g}	0.519^{cd}
Cv17B	0.145^{efg}	0.177^{bc}	1.504^{efg}	1.707^{cd}	0.782^{cd}	0.997b	0.314^{fg}	0.525^{c}

Cv17D	0.134^{g}	0.239^{a}	1.461^{fg}	2.180^{a}	0.765^{d}	1.200^{a}	0.290^{fg}	0.734^{a}
P-value	0.0000		0.0001		0.0280		0.0023	
F-value	8.71		5.87		2.60		3.93	
$LSD_{0.05}$	0.0234		0.1755		0.1748		0.0992	

*Column means followed by the same letter are not significantly different at $P \leq 0.05$, according to Fisher's least significant difference.

3.3.5 Effect of *Rhizobium leguminosarum* and *Meloidogyne enterolobii* application time stem diameter, number of nematode eggs in root and total nematodes

For all three measured variables, stem diameter, number of nematode eggs in root and total nematodes, there were highest when *M. enterolobii* was applied first and lowest when *R. leguminosarum* was applied first, but the two were not different from the simultaneous application (Table 3.8).

Table 3.8: Time of *Rhizobium leguminosarum* and *Meloidogyne enterolobii* application on plant and nematode variables.

Treatment	Stem diameter	Nema Egg roots	Total nematodes
Nematode first	0.168[a]	2.796[a]	3.903[a]
Both organisms	0.158[ab]	2.431[ab]	3.787[ab]
Rhizobium first	0.155[b]	2.273[b]	3.541[b]
P-value	0.035	0.024	0.032
F-value	3.45	3.83	3.53
$LSD_{0.05}$	0.0107	0.3866	0.2746

*NemaEgg -Nematode eggs in the roots. Column means followed by the same letter are not significantly different at $P \leq 0.05$, according to Fisher's least significant difference.

3.3.6 Effects of variety and season on rhizobium variables

There were clear differences in the effects of season on cowpea nodulation among different varieties (Table 3.9). Variety Cv17A had the highest of all the three nodulation variables in winter, and the least during summer (Table 3.9). Variety Cv17D was not statistically different from the best performing variety Cv17A in winter. Cv17D had the highest nodulation variables during summer (Table 3.9). Variety Cv17I was generally the lowest performing in all two seasons, with all other variables not statistically different from each other (Table 3.9).

Table 3.9 Interaction of variety type and season on rhizobium variables

	Season					
	Winter	Summer	Winter	Summer	Winter	Summer
Variety	Active nodules		Non-active nodules		Position of nodules	
Cv17C	0.225^{e}	0.926^{b}	0.236^{de}	1.146^{a}	0.165^{fg}	0.525^{ab}
Cv17A	0.549^{cd}	0.751^{bc}	0.473^{cd}	0.536^{c}	0.383^{bcde}	0.353^{cde}
Cv17F	0.196^{e}	0.971^{ab}	0.239^{de}	0.862^{b}	0.273^{efg}	0.448^{abcd}
Cv17I	0.099^{e}	0.624^{c}	0.124^{e}	0.965^{ab}	0.117^{g}	0.393^{abcde}
Cv17B	0.269^{de}	0.974^{ab}	0.436^{cd}	0.848^{b}	0.319^{def}	0.482^{abc}
Cv17D	0.513^{cd}	1.249^{a}	0.280^{cde}	1.163^{a}	0.264^{efg}	0.545^{a}
P-value	0.0496		0.0001		0.116	
F-value	2.28		5.47		3.07	
$LSD_{0.05}$	0.2834		0.2817		0.1592	

* Column means followed by the same letter are not significantly different at $P \leq 0.05$, according to Fisher's least significant difference.

3.3.7 Effect of inoculum application time and variety on active nodules and position of nodules

Variety Cv17D was the best performing variety irrespective of the inoculation time of *R. leguminosarum* and *M. enterolobii* (Table 3.10), whereas Cv17I was the poor performing variety (Table 3.10). Inoculating variety Cv17A with Rhizobium before nematode inoculation gave the highest active nodules when compared to inoculating it with nematode first or simultaneous inoculation, but the inoculation time did not affect the position of the nodules (Table 3.10). Varieties, Cv17I and Cv17D had the same number of active nodules and position of these nodules

irrespective of the inoculation time, whereas Cv17C, and Cv17B had similar number of active nodules at all three nodulation times but lower nodule position scores whem the two were applied simultaneously (Table 3.10). Only variety Cv17F had higher nodule position scores when the two microorganisms were inoculated simultaneously than when inoculated separately (Table 3.10).

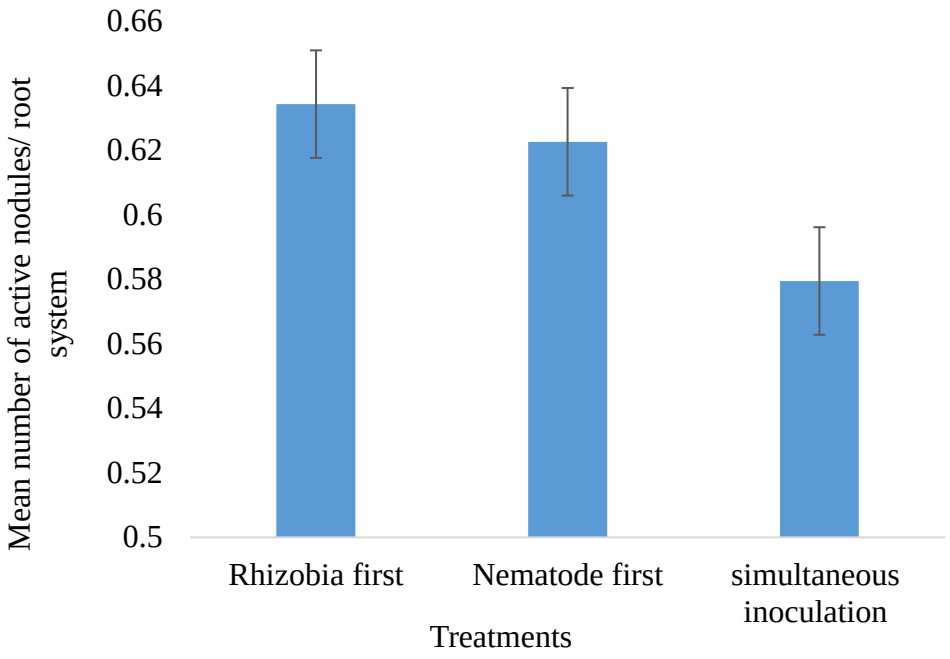

Figure 3.3: Mean number of active nodules on cowpeas, with *R. leguminosarum* first. *M. enterolobii* first and simultaneous inoculation of *R. leguminosarum* and *M. enterolobii*.

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Table 3.10 Effect of treatments on the number of active nodules and position of nodules.

	R	N	B	R	N	B
Variety	Active nodules			Position of nodules		
Cv17C	0.709^{abcd}	0.641^{abcde}	0.376^{def}	0.414^{abcd}	0.401^{abcd}	0.213^{e}
Cv17A	0.941^{a}	0.502^{cdef}	0.505^{cdef}	0.372^{abcde}	0.319^{bcde}	0.414^{abcd}
Cv17F	0.497^{cdef}	0.551^{bcdef}	0.703^{abcd}	0.289^{cde}	0.276^{cde}	0.517^{a}
Cv17I	0.216^{f}	0.356^{ef}	0.513^{cdef}	0.211^{e}	0.325^{abcde}	0.228^{de}
Cv17B	0.666^{abcde}	0.709^{abcd}	0.489^{cdef}	0.432^{abc}	0.482^{ab}	0.289^{cde}
Cv17D	0.777^{abc}	0.977^{a}	0.891^{ab}	0.379^{abcde}	0.409^{abcd}	0.427^{abc}
P-value		0.0491			0.0380	
F-value		1.91			2.00	
$LSD_{0.05}$		0.3471			0.1988	

* R -Rhizobium first. N-Nematode first. B-both nematodes and rhizobium. Column means followed by the same letter are not significantly different at $P \leq 0.05$, according to Fisher's least significant difference.

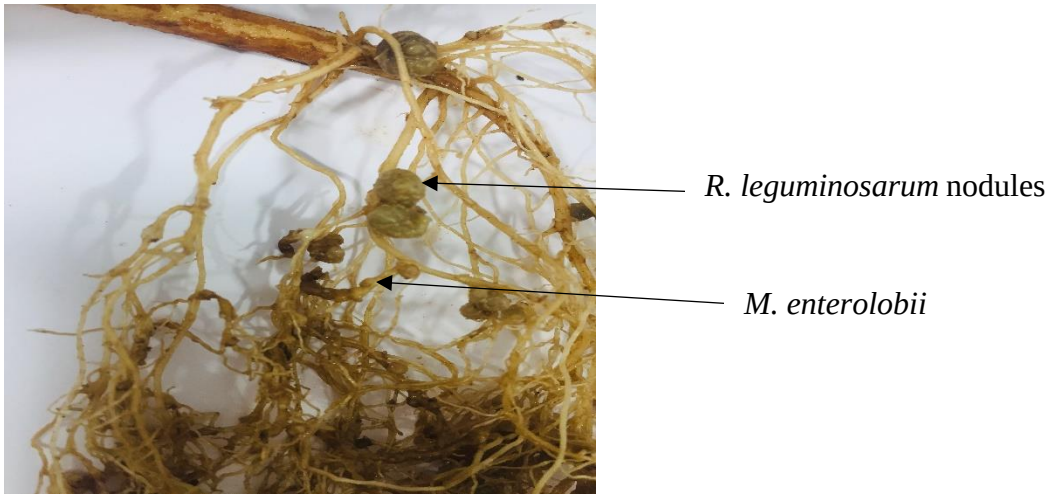

Figure 3.4. *R. leguminosarum* nodules and *M. enterolobii* galls occupy the same space on cowpea roots

3.4 Discussion

In this study, the relationship between two organisms is being investigated and *Rhizobium leguminosarum* for its effectiveness in nematode management. When *M. enterolobii* was applied first the total number of nematodes eggs in the roots was high and the total number of nematodes were the highest compared to when there was simultaneous inoculation and the inoculation of *R. leguminosarum* first. This could have been because the nematodes have already occupied the rhizosphere in which the rhizobia were not able to supply the host plant with nitrogen. Khan, Mohidin, Khan & Ahamad, (2016) stated that the invasion of nodules by nematodes results in a decreased population of bacteroids and the contents of leghemoglobin in nodules, the development of the nodule and reproduction of rhizobia get affected.

When *R. leguminosarum* was applied first we observed a small stem diameter and a lower number of total nematodes and nematode eggs in the roots. This result indicates that the application of *R. leguminosarum* can limit the nematodes' penetration and reproduction in the rhizosphere. Veken. *et al*. (2020) reported that this behavior could be because of competition between the two micro-

organisms, where fewer nutrients were available for the developing female nematodes which then affects the development and reproduction of the nematodes.

3.4.1 Effect of *Rhizobium leguminosarum* and *Meloidogyne enterolobii* application time and season on nematode variable

Our results revealed that during the two seasons (winter and summer) the activity of nematodes was greater during the summer season experiment than the winter season. This statement is supported by by Ahmed, Islam, Hamada, Shi, Shiming & Jingy *et al*., (2019), who reported that temperatures play a vital role in the life cycle of nematodes. Ahmed *et al*., 2019 further stated that the nematodes become active when the soil temperature is at 18-32 ^{o}C, which leads to high gall formation. In the paper, Ahmed *et al*. (2019) also reported that high temperatures caused a loss of resistance in host plants like tomato plants. This study showed that during the summer experiment the activity of nematodes increased when the simultaneous inoculation of *R. leguminosarum* and *M. incognita* was done. This disagrees with a study conducted by Veken *et al*. (2020) who demonstrated that simultaneous inoculation of *R. etli* and *M. incognita* reduced the reproduction of *M. incognita.* The difference in these results could have been because the number of nematodes was more than the number of rhizobia in this study. However, this could have not been the actual reason behind the high number of nematodes and low number of nodules. Khan *et al*. (2012) in his stated that a reduction in mung bean nodulation was a result of increased inoculation levels of nematodes which affected the number of nodules.

Another possible reason for an increased number of nematode eggs, when the simultaneous inoculation was done, could be that in the process of both micro-organisms establishing feeding sites, *M. enterolobii* attacked *R. leguminosarum* in the process due to the competition for space in

the rhizosphere. This is supported by Costa, Ng & Mathesius (2021) who stated that bacterial nodules are attacked at an early stage by nematodes and the nodules become galls, unlike when the attack takes place at a later stage the bacteria nodules remain as nodules.

3.4.2 Effect of the different seasons on nematode variables

As mentioned earlier in the discussion, nematodes are active and reproduce more in warmer temperatures. Different cowpea varieties responded differently to the nematode damage. This agrees with a study by Adomako, Kingsley, Danso, Sackey, Bismark & Kankam, (2016) who reported that the different response of cowpeas to nematode populations is variety dependent as none of the varieties used suppressed the population of nematodes. He further stated that some commonly used cowpea varieties have various degrees of resistance to one or more species of nematodes.

3.4.3 Effect of different seasons on plant growth variables

Cowpeas are legumes that are best suited for warm weather and are known to be drought tolerant. The study conducted shows that cowpea plants grew better in summer than in winter. The evidence gathered included thicker stem diameters, greater plant heights, higher fresh root and dry shoot weights across all cowpea varieties, as well as a higher growth rate. Olusanya, Gideon, Emmanuel, Akinjide, Akash & Ademayowa (2016) stated that the yield and characteristics of cowpea are affected by temperature because the final yield is determined by the growth temperature in that specific environment. In an area that has an average temperature of 25 ^{o}C, cowpeas tend to show optimum growth (Gomes, Rodrigues & Antonio, 2020).

3.4.4 Effects of inoculation time on plant and nematode variables

Different times of application of *R. leguminosarum* and *M. enterolobii* had different effects on plant diameter, number of nematode eggs in the roots and the total nematodes. The application of *M. enterolobii* first resulted in a thicker stem diameter compared to the simultaneous application of *R. leguminosarum* and *M. enterolobii* and the pre-inoculation of *R. leguminosarum* My results disagree with the findings of Tijjani & Atungwu (2017) who stated that an increase in the stem diameter is caused by the transportation and uptake of water and nutrients which depend on healthy roots. Tijjani et al. (2017) further stated that a thicker stem diameter is caused by the translocation of water and nutrients to the shoots. A nematode-infected root system shows a reduced size of stem diameter when compared to a non-nematode infected plant stem. In our case, it could have been that our cowpea varieties have resistance or tolerant genes to *M. enterolobii*. Osei, Gowen, Pembroke, Brandenburg & Jorden (2010) reported that legume plants such as cowpeas contain several chemicals which potentially influence the behavior of the nematodes. Some of the varieties used in this experiment might have properties such as the plant producing proteins that inhibit the survival of the nematode in the root system. Some varieties could be tolerant to nematode damage and be able to produce a healthy stem and produce good yields. However, when *R. leguminosarum* was applied first we observed thinner stem compared to when *M. enterolobii* was applied first, this could have been because of the interaction between *R. leguminosarum* and *M. enterolobii* occupying the same space in the rhizosphere (Figure 3.4). The activity of the two micro-organisms has influenced the growth rate of the host plant but more research needs to be done for my clarity of what really happens. The occupation of both organisms on the roots of cowpeas did not affect the yield in our study. Anter, Amin, Ashoub & El-nuby, (2014) support our results, they reported that the inoculation of plant growth promoting rhizobium species alone or together in plants infested by *M. javanica* increased plant growth.

3.4.5 Effect of variety on rhizobium variables

Legumes could establish symbiotic relationships with bacteria that can fix atmospheric nitrogen gas. The bacteria including rhizobia which infect the roots and induce root nodules of legumes where nitrogen fixation takes place (Peix, Bahena, Velazquez & Bedmar, 2015). When nitrogen fixation takes place inside the nodules it forms a pink color which shows a sign of efficient symbiosis and a white color indicating ineffective symbiosis (Peix *et al*., 2015). In our results, the highest number of active nodules and non-active nodules were recorded during the summer season experiment. This is because cowpeas are said to be warm seasonal crops because during the summer seasons optimum growth is obtained. There was greater growth during the summer season than during the winter season (Table 3.7), this explains the high number of active nodules during the summer season which means that there was efficient symbiosis in the rhizosphere. Although each variety had different numbers of active nodules on their roots due to different varieties having different properties. There was still efficient symbiosis in all cowpea varieties despite the different numbers of active nodules.

3.4.6 Effect of inoculation time and variety on rhizobium variables

Certain factors affect the competitiveness of rhizobia such as the soil type and physiochemical properties and the method of inoculation used (Rathi. Tak. Bissa. Chouhan. Ojha & Adhikari. 2018).

Different varieties reacted differently to each treatment and there were differences in the number of active and non-active nodules on their root systems. The graph (figure 3.3) in the results section shows that the number of active nodules was high when *R. leguminosarum* was applied first and low when there was simultaneous inoculation. According to Elhady, Hallmann, and Heuer (2020),

once rhizobia cells have established themselves in the roots and formed nodules, P. penetrans does not affect the nodules. The reason for this may be that the roots have already completed the acquisition process with *B. japonicum* before the inoculation with *P. penetrans* took place.

3.4 Conclusion

Different cowpea varieties respond differently to nematode infection. Inoculating legume seeds with rhizobia assists with plant growth under nematode-infected soils because of the symbiotic relationship the legume crop has with the rhizobia. We noticed that an organism that gets to the roots of the host plant first dominates the root system leaving no space for the other micro-organism to establish its feeding site. This becomes a challenge in situations where the fields have a high population of root-knot nematodes when wanting to implement a biological control method using rhizobia. Pre coating of seeds with rhizobia is recommended to reduce the damage that is caused by root knot nematodes but keeping in mind the species of the rhizobia and nematode.

More studies on the relationship of the two micro-organism and how they impact each other once they occupy the root system of the host plant need to be done.

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CHAPTER 4

SUMMARY, SIGNIFICANCE OF FINDINGS, FUTURE RESEARCH, AND CONCLUSIONS

4.1 Summary

This study assessed the relationship between commercial *R. leguminosarum* and *M. enterolobii*, to establish their effects on the host plant *Vigna unguiculata* varieties, under greenhouse conditions in two growing seasons (summer and winter), for future use of rhizobia as a biological control against root-knot nematodes *Meloidogyne* spp. There was a clear difference in the infectivity of both organisms under the two different growing seasons. Different varieties responded differently to both microorganisms. During the summer season, there was a high number of nematode galls and less active rhizobium nodules, which shows that *M. enterolobii* was more active during warmer conditions than cold conditions. The host plant managed to produce mature pods under the infestation of nematodes in both seasons, although the growth rate differed and showed that the rhizobium was able to work with the host plant to tolerate/outcompete the damage of the nematodes on the host plant.

4.2 Significance of the study

This study showed that rhizobium does assist with the reduction of the level of nematode damage on cowpea varieties. Under nematode-infested soils plant growth was able to take place and other varieties were able to produce pods. My study demonstrated that the use of rhizobium in legume plants to control nematode damage is efficient because it promotes greater growth and reduce nematode damage/infection. This study will contribute to improved production of legume crops

that are host to root-knot nematodes and most importantly to small-scale farmers in Southern Africa who do not have the capital to invest in nematicides..

4.3 Future research

Rhizobium leguminosarum assisted cowpea varieties in promoting plant growth under soils infested with nematodes, but the relationship between the two micro-organisms *R. leguminosarum* and *M. enterolobii* is not yet fully understood or known. Studies looking deep into the biochemical relationship between the two micro-organisms once in the rhizosphere of the plant need to be done. Although *Rhizobium leguminosarum* promoted growth in cowpea varieties, it does not mean that other legume crops will also react similarly, hence more studies need to be performed on different crops to explain and show the effectiveness of rhizobacteria as a means of biological control of root-knot nematodes. A study on the lifecycle of *Meloidogyne enterolobii* must be conducted, to know which stages are most infective and during which seasons to know when to implement biological control.

4.4 Conclusion

Cowpea is a host plant to root-knot nematodes, *Meloidogyne* spp. Commercial *R. leguminosarum* was efficient in promoting plant growth under nematode-infested soils. The occurrence of both nematode galls and rhizobium nodules on the same root systems was observed in our study. Different growing seasons have different effects on the activity of both *M. enterolobii* and *R. leguminosarum*. With the results obtained from our study, we have seen that rhizobium has the potential to reduce the nematode infestation, but its effectiveness depends on favorable conditions for both the plant and the rhizobium. More studies should be conducted on the potential use of rhizobium as potential biological control for root knot nematodes.

APPENDICES

Appendix 3.1: Shapiro-Wilk normality test for all plant and nematode variables

Variable	N	W	P
Diameter	174	0.9441	0.0000
Height	177	0.7485	0.0000
Dry shoot	177	0.8945	0.0000
Fresh root	177	0.8326	0.0000
NemaJ2roots	175	0.6834	0.0000
Nemaeggsroots	175	0.5884	0.0000
NematJ2soil	180	0.9040	0.0000
Total nematodes	180	0.8521	0.0000
Reproductive potential	177	0.7009	0.0000

Appendix 3.2: Shapiro-Wilk test for rhizobium variables

Variable	N	W	P
Active nodules	177	0.7825	0.0000
Non-active nodules	177	0.7551	0.0000
Position of nodules	177	0.8153	0.0000
Total nodules	177	0.8565	0.0000

Appendix 3.3: Analysis of variance for plant diameter.

Source	DF	SS	MS	F	P
Replication	4	0.00103	0.00026		
Season	1	0.10495	0.10495	127.51	0.0000
Variety	5	0.04691	0.00938	11.40	0.0000
Treatment	2	0.00567	0.00284	3.45	0.0348
Season*Variety	5	0.03586	0.00717	8.71	0.0000
Season*Treatment	2	0.00028	0.00014	0.17	0.8455
Variety*Treatment	10	0.00554	0.00055	0.67	0.7472
Season*Variety*Treatment	10	0.01017	0.00102	1.24	0.2742
Error	134	0.11029	0.00082		
Total	173				

Appendix 3.4: Analysis of variance for plant height

Source	DF	SS	MS	F	P
Replication	4	0.85354	0.21339		
Season	1	6.96265	6.96265	133.15	0.0000
Variety	5	1.43053	0.28611	5.47	0.0001
Treatment	2	0.04904	0.02452	0.47	0.6267
Season*Variety	5	1.53391	0.30678	5.87	0.0001
Season*Treatment	2	0.03580	0.01790	0.34	0.7107
Variety*Treatment	10	0.43699	0.04370	0.84	0.5951
Season*Variety*Treatment	10	0.34073	0.03407	0.65	0.7672
Error	137	7.16400	0.05229		
Total	176				

Appendix 3.5: Analysis of variance for fresh-root mass

Source	DF	SS	MS	F	P
Replication	4	0.12079	0.03020		
Season	1	2.55303	2.55303	49.21	0.0000
Variety	5	2.34277	0.46855	9.03	0.0000
Treatment	2	0.11013	0.05506	1.06	0.3488
Season*Variety	5	0.67430	0.13486	2.60	0.0280
Season*Treatment	2	0.06012	0.03006	0.58	0.5616
Variety*Treatment	10	0.72786	0.07279	1.40	0.1851
Season*Variety*Treatment	10	0.60728	0.06073	1.17	0.3159
Error	137	7.10743	0.05188		
Total	176				

Appendix 3.6: Analysis of variance for dry shoot mass

Source	DF	SS	MS	F	P
Replication	4	0.06555	0.01639		
Season	1	3.17951	3.17951	190.28	0.0000
Variety	5	0.53276	0.10655	6.38	0.0000
Treatment	2	0.08350	0.04175	2.50	0.0860
Season*Variety	5	0.32805	0.06561	3.93	0.0023
Season*Treatment	2	0.00859	0.00430	0.26	0.7737
Variety*Treatment	10	0.24975	0.02498	1.49	0.1476
Season*variety*Treatment	10	0.25874	0.02587	1.55	0.1288
Error	137	2.28926	0.01671		
Total	176				

Appendix 3.7: Analysis of variance for nematode J2 in root

Source	DF	SS	MS	F	P
Replication	4	5.261	1.315		
Season	1	214.926	214.926	196.05	0.0000
Variety	5	11.879	2.376	2.17	0.0614
Treatment	2	3.438	1.719	1.57	0.2123
Season*Variety	5	6.292	1.258	1.15	0.3384
Season*Treatment	2	7.396	3.698	3.37	0.0372
Variety*Treatment	10	14.108	1.411	1.29	0.2439
Season*Variety*Treatment	10	7.366	0.737	0.67	0.7489
Error	135	147.998	1.096		
Total	174				

Appendix 3.8: Analysis of variance for nematode eggs in root

Source	DF	SS	MS	F	P
Replication	4	3.488	0.872		
Season	1	145.936	145.936	133.63	0.0000
Variety	5	15.716	3.143	2.88	0.0168
Treatment	2	8.372	4.186	3.83	0.0240
Season*Variety	5	15.264	3.053	2.80	0.0195
Season*Treatment	2	12.988	6.494	5.95	0.0033
Variety*Treatment	10	9.050	0.905	0.83	0.6017
Season*Variety*Treatment	10	6.362	0.636	0.58	0.8260
Error	135	147.429	1.092		
Total	174				

Appendix 3.9: Analysis of variance for nematode J2 in soil

Source	DF	SS	MS	F	P
Replication	4	11.790	2.947		
Season	1	116.517	116.517	70.24	0.0000
Variety	5	6.134	1.227	0.74	0.5950
Treatment	2	3.985	1.992	1.20	0.3039
Season*Variety	5	5.674	1.135	0.68	0.6363
Season*Treatment	2	0.604	0.302	0.18	0.8338
Variety*Treatment	10	18.830	1.883	1.14	0.3404
Season*Variety*Treatment	10	10.167	1.017	0.61	0.8009
Error	140	232.229	1.659		
Total	179	405.929			

Appendix 3.10: Analysis of variance for total nematodes

Source	DF	SS	MS	F	P
Replication	4	1.611	0.4026		
Season	1	71.046	71.0460	122.80	0.0000
Variety	5	6.112	1.2224	2.11	0.0674
Treatment	2	4.090	2.0451	3.53	0.0318
Season*Variety	5	7.061	1.4123	2.44	0.0373
Season*Treatment	2	1.693	0.8467	1.46	0.2350
Variety*Treatment	10	10.685	1.0685	1.85	0.0578
Season*Variety*Treatment	10	7.721	0.7721	1.33	0.2178
Error	140	81.000	0.5786		
Total	179	191.020			

Appendix 3.11: Analysis of variance for reproductive potential

Source	DF	SS	MS	F	P
Replication	4	3.001	0.750		
Season	1	138.198	138.198	185.73	0.0000
Variety	5	9.320	1.864	2.51	0.0332
Treatment	2	5.458	2.729	3.67	0.0281
Season*Variety	5	8.924	1.785	2.40	0.0404
Season*Treatment	2	9.560	4.780	6.42	0.0022
Variety*Treatment	10	10.403	1.040	1.40	0.1874
Season*Variety*Treatment	10	1.913	0.191	0.26	0.9890
Error	137	101.941	0.744		
Total	176				

Appendix 3.12: Analysis of variance for active nodules

Source	DF	SS	MS	F	P
Replication	4	0.5944	0.1486		
Season	1	16.5877	16.5877	107.64	0.0000
Variety	5	4.1783	0.8357	5.42	0.0001
Treatment	2	0.0998	0.0499	0.32	0.7238
Season*Variety	5	1.7591	0.3518	2.28	0.0496
Season*Treatment	2	0.0482	0.0241	0.16	0.8553
Variety*Treatment	10	2.9366	0.2937	1.91	0.0491
Season*Variety*Treatment	10	0.5347	0.0535	0.35	0.9663
Error	140	21.5744	0.1541		
Total	179	48.3133			