EFFECT OF USING *Trichoderma asperellum* A BIOCONTROL AGENT AGANIST ROOT KNOT NEMATODES ON NODULATION, GALLING AND GROWTH PARAMETERS OF FRENCH BEANS IN KENYA

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DECLARATION

I, Esther Waithira Kamau, declare that this thesis is my original work and has not been presented for the award of a degree in any other university or any other award.

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DEDICATION

This thesis is dedicated to my husband George N. Kareithi and our children Newton Kareithi, Diana Wangui and Ezra Kamau for their support, prayers and encouragement during the pursuit of this degree.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
BNF	Biological Nitrogen Fixation
BTB	Bromothymol Blue
CAN	Calcium Ammonium Nitrate
CMD	Cornmeal Dextrose Agar
CR	Congo Red
CWDEs	Cell Wall Degrading Enzymes
DAP	Diamonium Phosphate
DNA	Deoxyribonucleic acid
FYM	Farm Yard Manure
HCDA	Horticultural Crop Development Authority
J2	Infective second juvenile stage of root-knot nematodes
MRL	Maximum Residual Level
NAFIS	National Farmers Information Service
PDA	Potato Dextrose Agar
RGH	*Rhizobium* from the greenhouse
RKN	Root-knot Nematodes
RNA	Ribonucleic acid
T1-T4	Treatment 1-Treatment 4
UNEP	United Nation Environmental Programme
YMA	Yeast Mannitol Agar

ABSRACT

Trichoderma species have been widely reported worldwide as biocontrol agents of plant pathogens and growth promoters. *Trichoderma asperellum* has been fronted as a biocontrol agent of root-knot nematodes on tomato and French beans in Kenya. However research on the effect of *T. asperellum* on nodulation particularly in French beans is still lacking. Therefore there is a need to determine the effect of *Trichoderma* spp. on nodulation. A negative effect could counter the positive effect since *Rhizobium* species is very important for nitrogen fixation. The objective of this study was therefore to determine the effect of *T. asperellum* on nodulation in French beans. A total of five treatments were applied. These were *T. asperellum* at low rate, at medium rate, and at high rate. The other treatments were a standard biological nematicide, Bionematon and an untreated control. Greenhouse experiments were carried out to assess nodulation under controlled conditions. Nodulation index was determined using the nodule size, color, nodule number and nodule biomass. The biomass of the roots and shoots were also assessed as well as root collar diameter. The presence of root-knot nematodes (RKN) within the treatments was confirmed using the Baermann's method in the soil and the presence of galls in the roots. Data was analyzed using analysis of variance (ANOVA) and the means were separated using Tukey's test at 5 % level. The *in vitro* research output based on the laboratory experiments indicates that *T. asperellum* inhibits the growth of *Rhizobium* spp. by between 50.13 - 100 %. Greenhouse experiments showed that treatment with *T. asperellum* reduced the galling in French beans significantly and had no effect on the investigated plant growth parameters. There was a significant difference in the nodule number but no significant difference in the nodule biomass. This is probably due to the fact that, although the nodules increased in numbers in the control and in low level of *Trichoderma*, the nodule size reduced as a result of the high level of galling that occurred in absence of nematode control. In the light of these findings, there is need to evaluate the effect of *Trichoderma* spp. on nodulation of other legumes and on the growth of other *Rhizobium* spp. Since nodulation in French beans was reduced by application of *T. asperellum*, there is need to determine if nitrogen fixation is compromised and whether there in need to revise the amounts of nitrogenous fertilizers applied where the soil is treated with *Trichoderma*.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

French beans (*Phaseolus vulgaris* L.) originated from South and North America and were introduced in Kenya from Europe (www.Unispice.com, - accessed on 3rd July 2010). The common cultivars of French beans grown in Kenya are; Teresa morgan, Espadia, Paulista, Bakara, Cupvert, Claudia, Super monel, Gloria, Tokai, Tonivert, Pekera, Amy, Samantha, Julia, Vernando, Bronco and Rexas. Other varieties are still on trial in Kenya. The leaves have three leaflets with a long petiole. The stalked flower head is in the form of a short raceme. The long pods vary in colour, depending on cultivar while the seeds may be black, white, red coloured or marbled. The growth habit is both dwarf and climbing type (HCDA, 2012).

The plant grows in a wide range of environments. The major production areas in Kenya are Athi River, Kirinyaga, Meru and Naivasha (HCDA, 1996). It is the most important export vegetable crop in Kenya, mainly exported to Europe, for instance in 2007 it earned the country approximately Kenya shillings 889.4 million (HCDA, 2007). However the production of French beans is declining due to production constraints such as diseases and pests, transport and marketing costs (Monda *et al*., 2003). However the demand for French beans is increasing worldwide. The major diseases of French beans are rusts, *Fusarium* wilt and blight while pests include nematodes, bean flies, and white flies. Over use of fungicides is common as the French beans are sprayed twice a week to control diseases and pests (Monda *et al*., 2003). The over usage of chemical pesticides present

special challenges especially for the export market of locally produced French beans exported to European market which is subject to maximum residual levels (MRL). As such there has been a concerted effort to replace these chemical pesticides with biological agents.

Trichoderma spp. have been used as biocontrol agents against soil-borne pathogens such as *Fusarium, Pythium* and *Rhizoctonia* spp. in French beans (Kariuki *et al*., 2010) among other crops. *Trichoderma* spp. is also being used in the management of soil-borne diseases in French beans particularly for the export market. It is hypothesized that *Trichoderma* spp. acts by antibiotic production, mycoparasitism, production of cell wall-degrading enzymes and competition for nutrients or space to achieve biocontrol of the pathogens (Zeilinger and Omann, 2007; Vinale *et al*., 2008).

1.2 Problem statement

French beans are leguminous plants with the ability to fix free nitrogen from the air into nitrates in symbiosis with the *Rhizobium* bacteria in their root nodules. Nitrates help in the photosynthetic activity of plants eventually leading to high yields and also avails nitrates for subsequent crops (Emerich and Hari, 2009). If nodulation is compromised, then the farmer may need to spend more money buying nitrogenous fertilizers to boost crop yield. French beans are an important export horticultural crop to United Kingdom and France and its demand has been increasing annually (HCDA, 2010). Any measures that could promote an increase in production would benefit not only the farmer but also the country at large in terms of foreign exchange earnings.

French beans are attacked by pests such as root-knot nematodes (RKN) which constitute a very significant threat to French bean production in Kenya (Kariuki *et al*., 2011). Root-knot nematodes can be managed through crop rotation and only recently through biological control. The use of the conventional nematicides is expensive to the farmers and at the same time detrimental to the environment. Furadan (carbofuran), was one of the previously used nematicides but was withdrawn from the market due to residues found in the French beans (Monda *et al*., 2003).

1.2.1 Justification

Trichoderma spp. also has the potential to control some of the fungal diseases as well as root knot nematodes in French beans. However, even if its use was to be successful, there has never been any documented study on the effect of *Trichoderma* spp. on nodulation and growth of French beans. This study is therefore very relevant so that the effect of commercially available *Trichoderma* spp. on nodulation and growth of French beans is established early enough.

1.3 Research questions

i) Which *Rhizobium* spp. isolates infect French beans in Mwea, Kenya

ii) What is the *in vitro* interaction between *Trichoderma* spp. and *Rhizobium* spp.?

iii) Does the use of *Trichoderma* spp. a biocontrol agent against nematodes in French beans affect nodulation?

iv) Does the use of *T. asperellum* a biocontrol agent against root knot nematodes affect growth parameters in French beans?

1.4 Hypotheses

i) There are no different *Rhizobium* spp. isolates that infect French beans in Mwea, Kenya.

ii) *Trichoderma asperellum* does not affect the growth of *Rhizobium* spp. *in vitro*.

iii) *Trichoderma asperellum* does not affect nodulation in French beans when used as a biocontrol agent of root-knot nematodes.

iv) *Trichoderma asperellum* does not affect growth parameters in French beans when used as a biocontrol agent of root-knot nematodes.

1.5 Objectives

1.5.1 General objective

To determine the effect of *T. asperellum* on nodulation, galling and growth of French beans when used as a biocontrol agent against root knot nematodes (*Meloidogyne* species).

1.5.2 Specific objectives

i) To isolate and identify the *Rhizobium* spp. isolates infecting French beans in Mwea, Kenya.

ii) To determine the effect of *T. asperellum* on the growth of *R. leguminosarum in vitro*.

iii) To assess the effect of *T. asperellum* on nodulation, galling and growth of French beans when used as a biocontrol agent of root-knot nematodes.

iv) To assess the effect of *T. asperellum* on growth parameters in French beans when used as a biocontrol agent of root-knot nematodes.

1.6 Significance of the study

The use of *Trichoderma* spp. to control nematodes and the fungal pathogens such as *Pythium* spp., *Rhizoctonia* spp. and *Fusarium* spp. will go a long way in assisting farmers to realize effective and efficient French bean production. *Trichoderma* spp. acts both as a biological fungicide as well as a nematicide without residual toxic effects in the soil. This is unlike most of the fungicides and chemical nematicides available in the market. The use of these chemicals in crop production has resulted in chemical residues which have led to the rejection of the Kenyan horticultural products particularly in the European market. Nodulation in French beans and generally in legumes is important since it avails nitrates to the plant without the use of inorganic nitrogen fertilizers. A plant which has sufficient supply of nitrogen grows with vigor and eventually registers higher crop yields.

Since previous study has shown *Trichoderma* spp. have proved to be an efficient biological control agent of nematodes and soil borne pathogens in the production of French beans, it is important to establish the interaction between *Trichoderma* spp. and *Rhizobium* spp. It is therefore important to determine the effect of *Trichoderma* spp. on nodulation in French beans. A negative effect of *Trichoderma* spp. on nodulation may require the review of the amount of nitrogenous fertilizer used by growers for top-dressing French beans and the cost associated with this.

Biological nitrogen fixation (BNF) reduces the levels of inorganic nitrogen fertilizers that are applied and thus reduces the cost of production. The cost of inorganic nitrogen fertilizers have been escalating with every season. Excess amounts of inorganic nitrogenous fertilizers applied to crops may eventually find their way into water bodies through leaching and also into the atmosphere. This results in negative effects to the environment. The excess amounts of nitrogen in water bodies results in eutrophication. This reduces availability of oxygen for the flora and fauna in aquatic systems and blocks navigation in affected water bodies. The use of excess amounts of inorganic nitrogen fertilizers in agriculture may add excess nitrous oxide gases in the atmosphere, resulting in global warming.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Trichoderma* spp.

Trichoderma spp. are free living endophytic fungi present in all soils and root ecosystems. They are most prevalent and easy to culture. Many species in this genus can be characterized as opportunistic avirulent plant symbionts (Harman *et al*., 2004). The genus comprises a great number of fungal strains that act as biological control agents, the antagonistic properties of which are based on the activation of multiple mechanisms (Benitez *et al*., 2004). *Trichoderma asperellum* parasitizes a large variety of phytopathogenic fungi. The mycoparasitic activity of *T. asperellum* depends on the secretion of complex mixtures of hydrolytic enzymes like protease able to degrade the host cell wall and host (Xiaoxue *et al*., 2013).

This genus has been extensively studied owing to its ability to rapidly colonize substrates (Grondona *et al*., 1997), inducement of systemic acquired resistance in plants (Enkerly *et al*., 1999), their potential for promoting plant growth (Inbar *et al*., 1994) and antagonistic activity against a wide range of plant pathogenic fungi (Paulitz and Belanger, 2001). The inhibitory effect of their antibiotics and cell wall degrading enzymes (CWDEs) against many plant pathogens is often cited as important aspects of their antagonistic activity (Sivasithamparam and Ghisalberti, 1998).

The highly active nature and diversity of *Trichoderma* spp. enzymatic systems include glucanases, chitinases, proteases, lipases, esterases and DNAses (Benitez *et al*., 1998

Lorito, 1998,) which have led to their successful use in environmental and industrial biodegradation (Van Wyk and Mohulatsi, 2003). Applications derived from the use of these enzymes are related to their antifungal effect against phytopathogenic fungi containing α-1, 3-glucan in their cell wall, like *Fusarium oxysporum* (Howel, 2003).

Trichoderma spp. exert its biocontrol activity against fungal phytopathogens either indirectly, by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms and antibiosis or directly, by mechanisms such as mycoparasitism (Hafez *et al*., 2013). These indirect and direct mechanisms may act coordinately and their importance in the biocontrol process depends on the *Trichoderma* spp., the antagonized fungus, the crop plant, and the environmental conditions, including nutrient availability, pH, temperature, and iron concentration (Xiaoxue *et al*., 2013). Activation of each mechanism implies the production of specific compounds and metabolites, such as plant growth factors, hydrolytic enzymes, siderophores, antibiotics, carbon and nitrogen permeases. These metabolites can be either overproduced or combined with appropriate biocontrol strains in order to obtain new formulations for use in more efficient control of plant diseases and postharvest applications (Benitez *et al*., 2004).

There are 89 species in the genus *Trichoderma*. The cultures are typically fast growing at 25-30 °C, but will not grow at 35 °C (Harman, 2006). Depending on the type of media used, the colonies are transparent at first on cornmeal dextrose agar (CMD) media or white on richer media such as potato dextrose agar (PDA). Mycelium are not typically

obvious on CMD. Conidia typically form within one week in compact or loose tufts which are in shades of green, yellow or less frequently white. A yellow pigment may be secreted into the agar, especially on PDA (Harman, 2006). Some *Trichoderma* spp. produces a characteristic sweet or 'coconut' odor. Conidiophores are highly branched and thus difficult to define or measure but are often formed in distinct concentric rings. Main branches of the conidiophores produce lateral side branches (Hafez *et al*., 2013). The branches may be paired or not. Often, phialides arise directly from the main axis and near secondary branches closest to the main axis. All primary and secondary branches arise at or near 90° with respect to the main axis. The typical *Trichoderma* spp. conidiophores, with paired branches assume a pyramidal shape. Typically the conidiophore terminates in one or a few phialides (en.wikipedia.org - accessed on 10th December 2012).

Several strains of *Trichoderma* have been developed as biocontrol agents against fungal diseases of plants (Harman, 2006). *Trichoderma* spp. is known to produce a range of antibiotics such as trichodernin, trichodermol and harzianolide. They also produce cell wall degrading enzymes such as glucanase and chitinase which break polysaccharides and chitins destroying the cell wall (Howell, 2003; Kucuk and Kivanc, 2003). *Trichoderma* spp. has also been useful in the medical field. Cyclosporine A (CsA), a calcineurin inhibitor produced by the fungi *T. polysporum* and *Cylindrocarpon lucidum*, is an immunosuppressant prescribed in organ transplants to prevent rejection (Chong *et al*., 2009). *Trichoderma* spp. has also been put to industrial use, being a saprophyte adapted to thrive in diverse situations; it is known to produce a wide array of enzymes for example *T. longibratum* which is used to produce xylanase (Azin *et al*., 2007).

Trichoderma spp. has actually been investigated as biological control agents for over 80 years owing to their ability to antagonize plant pathogenic fungi (Howell, 2003). Fungi in the genus *Trichoderma* have been known since the 1920s for their ability to act as biocontrol agents against plant pathogens (Hafez *et al*., 2013). Recent advances demonstrate that the effects of *Trichoderma* spp. on plants, including induced systemic or localized resistance, are also very important. These fungi colonize the root epidermis and outer cortical layers and release bioactive molecules that cause walling off of the thallus. *Trichoderma harzianum* is also used to produce chitinase (Felse and panda, 1999).

Under influence of *Trichoderma* spp., the transcriptome and the proteome of plants are substantially altered. This may lead to increased plant growth and nutrient uptake. In maize, the increased growth response is genotype specific, and some maize inbreeds respond negatively to some strains. *Trichoderma* spp. is beginning to be used in reasonably large quantities in plant agriculture, both for disease control and yield increases (Harman, 2006). The studies of mycoparasitism also have demonstrated that the rich mixture of antifungal enzymes, produced by the fungi including chitinases and β-1, 3 glucanases actually are synergistic with each other, with other antifungal enzymes, and with other materials. The genes encoding the enzymes appear useful for producing transgenic plants resistant to diseases and the enzymes themselves are beneficial for biological control and other processes (Howell, 2003).

Some strains of *Trichoderma* spp. establish robust and long-lasting colonization of root surfaces and penetrate into the epidermis and a few cells below this level. They produce

or release a variety of compounds that induce localized or systemic resistance responses, and this explains their lack of pathogenicity to plants (Felse and Panda, 1999). These root-microorganism associations cause substantial changes to the plant proteome and metabolism. Plants are protected from numerous classes of plant pathogen by responses that are similar to systemic acquired resistance and rhizobacteria-induced systemic resistance.

Root colonization by *Trichoderma* spp. also frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients (Harman *et al*., 2004). Four strains of *T. asperellum* were used to control root rot disease caused by *Pythium myriotylum* which is a major threat to cocoyam cultivation in Cameroon (Mbargaa *et al*., 2012). The antagonistic and mycoparasitic potential of the *T. asperellum* strains were evaluated *in vitro* through dual culture and interaction tests. Subsequently, the ability of *T. asperellum* strains to protect cocoyam plant from *P. myriotylum* infection was tested *in vivo*. Results showed that all four *T. asperellum* strains were antagonistic to *P. myriotylum*, although differences were found among the strains. The growth of *P. myriotylum* was inhibited by more than 60 %. Furthermore, the four *T. asperellum* strains were aggressive mycoparasites of *P. myriotylum*. *In vivo* trials, pretreatment of cocoyam plants with the strains PR10 and PR11 of *T. asperellum* reduced *P. myriotylum* infection by 50 % (Mbargaa *et al*., 2012).

Studies aimed at elucidating the parasitic capabilities of *Trichoderma* spp. isolates on the RKN, *Meloidogyne javanica* and their biocontrol activities against the nematode were

carried out by Sharon *et al*. (2007).The results indicated that parasitism is probably an important mode of action and one of the initial steps of this process is attachment. The nematode's gelatinous matrix enabled fungal attachment and enhanced parasitic capabilities of the isolates, except *T. harzianum*, which could also utilize gelatinous matrix as a nutrient source. The gelatinous matrix has also been found to trigger proteolytic and chitinolytic enzyme production by the fungus. Mycoparasitism, one of the main mechanisms involved in the antagonistic activity of *Trichoderma* strains, depends on the secretion of complex mixtures of hydrolytic enzymes able to degrade the host cell wall. The antifungal activity of α-1, 3-glucanase enzyme able to degrade α-1, 3-glucans may have a role in mycoparasitic processes. An exo-α-1, 3-glucanase, namely AGN13.2, from the antagonistic fungus *T. asperellum* T32 has also been isolated (Sanz *et al*., 2004).

2.2 *Rhizobium* spp.

Bacteria belonging to the genera *Rhizobium, Mesorhizobium, Sinorhizobium, Bradyrhizobium* and *Azorhizobium* are collectively referred to as rhizobia (Gage, 2004). Over the years however, the term has come to be used for all the bacteria that are capable of nodulating and fix nitrogen in association with legumes that belong to a genus that was at one time part of the genus *Rhizobium* or closely related to it (Willems, 2006). *Rhizobium* spp. is a nitrogen-fixing bacterium that form root nodules on legume plants.

Most of these bacterial species are in the Rhizobiacae family in the alpha-proteobacteria. However a recent study has shown that there are many other rhizobial species. In some cases these new species have arisen through lateral gene transfer of symbiotic genes.

There are other non-rhizobial species present in these genera. For example in the genus *Rhizobium*, there is *Rhizobium radiobacter*, formerly known as *Agrobacterium tumefaciens*, which do not form nitrogen fixing symbiotic root nodules. Most of these belong to the order Rhizobiales, but several rhizobia occur in distinct bacterial orders of the proteobacteria. The *Agrobacterium* species is a non-symbiotic bacteria which is a closer relative of *Rhizobium* spp. than *Bradyrhizobium* species that nodulate soybean (www.rhizobia.co.nz//tazonomy/-accessed on 20th January 2013).

By the end of the 19th century, it was realized that atmospheric nitrogen was being assimilated through the root-nodules of legume plants (Gage, 2004). In 1888, Beijerinck reported isolation of the root nodule bacteria and established that they were responsible for the process of nitrogen fixation. He named these bacteria *Bacillus radicicola.* Later, Frank changed the name to *Rhizobium* with originally just one species, *R. leguminosarum*. In the beginning of the 20th century, extensive testing of nodulation of diverse legume hosts by different bacteria, led to the establishment of cross-inoculation groups, with rhizobia from one plant in a cross-inoculation group supposed to nodulate all other plants in the group. This concept was also used in rhizobial taxonomy, but was later abandoned as an unreliable taxonomic marker, partly because of aberrant cross-infection among plant groups (Willems, 2006).

A history into the studies on *Rhizobium* spp. reveals that in the early 1960s, bacteriologists started using a large diversity of morphological, nutritional and metabolic characteristics in numerical taxonomy studies. This demonstrated the relatedness of

Rhizobium and *Agrobacterium* and led to a clear distinction between the fast growing rhizobia with the latter group subsequently placed in a separate genus of the slow growing Bradyrhizobia. From the 80s on, with the introduction of more genetic characteristics, more diversity was discovered among the rhizobia. Their relationships with other groups of bacteria also became apparent. As a result there occurred a gradual increase in the number of genera (Young *et al*., 2003).

The current validly published binomial names for the rhizobia, consists of 98 species in 13 genera (Weir, 2012). Two reasons for this increase in the number of genera and species are that many different legume species have now been studied, in contrast to original efforts which emphasized those legumes that were important food and pasture species crops. Even now, only about 20 % of the18, 000 species and 57 % of about 650 genera of legume plants have been studied for nodulation (Weir, 2012). There is therefore a great potential of many more species and genera of rhizobia to be described. Another reason for increasing numbers of rhizobial species is the ongoing evolution of taxonomic research. This is due to new developments in the methods to study cell DNA and RNA which have led to a more detailed characterization resulting in phylogenetic and polyphasic classifications (www.rhizobia.co.nz//tazonomy/-accessed on 20th January 2013). Consequently this resulted to a separate genus for the fast-growing soybean rhizobia, renaming *R. fredii* as *Sinorhizobium fredii*, and proposal of a second species, *S. xinjiangense* (the original spelling *S. xinjiangensis*).

The genus *Mesorhizobium* was proposed for five rhizobial species (*R. loti, R. huakuii, R. ciceri, R. mediterraneum and R. tianshanense*) that are phylogenetically related and distinct from the large phylogenetic grouping that includes *Rhizobium, Agrobacterium* and *Sinorhizobium* (Jarvis *et al*., 1997). They are characterized by a growth rate intermediate between the fast and slow growing rhizobia. *Bradyrhizobium* was created for the slow-growing species *Rhizobium japonicum* (Willems, 2006). Originally the soybean-nodulating *B. japonicum* was the only species described, although it was recognized that slow-growing strains occur on various legume genera (Elkan and Bunn, 1992).

Agrobacterium is an artificial genus comprising plant-pathogenic species. The monophyletic nature of *Agrobacterium, Allorhizobium and Rhizobium* and their common phenotypic generic circumscription support their amalgamation into a single genus, *Rhizobium* (Gage, 2004). *Agrobacterium tumefaciens* was conserved as the type species of *Agrobacterium*, but the epithet radiobacter would take precedence as *R. radiobacter* in the revised genus. The proposed new combinations are *R. radiobacter, R. rhizogenes, R. rubi, R. undicola* and *R. vitis* (Young *et al*., 2003).

Only rhizobia that are specifically compatible with a particular species of legume can stimulate the formation of root nodules. The process by which the rhizobia produce nitrogen for the legume is called biological nitrogen fixation (BNF). Rhizobial inoculants are produced commercially in many countries. Inoculants contain rhizobia isolated from plant nodules and cultured artificially in the laboratory. In the laboratory, rhizobia are

grown on a special medium called yeast-mannitol agar (YMA). They are grouped in two main genera, the fast-growing *Rhizobium* spp. and the slow-growing *Bradyrhizobium* spp. When cultured on YMA, the *Rhizobium* spp. produces visible growth in two to three days. They produce an acid growth reaction, which can be detected by adding a pH indicator, bromthymol blue (BTB), to the medium (Beck *et al*., 1993).

Rhizobium spp. isolated from pea, bean, clover, alfalfa, chickpea, and leucaena are all fast growers. The *Bradyrhizobium* spp. takes six to eight days to produce visible growth on YMA and produce an alkaline reaction (www.ctahr.hawaii.education/bnf-accessed on 24th February 2013). The soybean and cowpea *Rhizobium* spp. are slow growers. *Rhizobium* spp. grown in the laboratory is shaped like short rods, as seen under the microscope. They measure 0.5 to 0.9 μm wide and 1.2 to 3.0 m long and they need a supply of oxygen to live. They can move using special thread-like structures called flagella (www.ctahr.hawaii.education/bnf accessed on 24th February 2013).

2.3 *Rhizobium*-legume relationship

Symbiotic nitrogen fixation by *Rhizobium* spp. in legume root nodules injects approximately 40 million tonnes of nitrogen into agricultural systems each year. In exchange for reduced nitrogen from the bacteria, the plant provides *Rhizobium* spp. with reduced carbon and all the essential nutrients required for bacterial metabolism. Symbiotic nitrogen fixation requires exquisite integration of plant and bacterial metabolism. Central to this integration are transporters of both the plant and the *Rhizobium* spp., which transfer elements and compounds across various plant membranes

and the two bacterial membranes. Although all legume-*Rhizobium* spp. symbioses have many metabolic features in common, there are also interesting differences between them (Brewina, 2010).

The relationship between *Rhizobium* spp. and legume plants is selective. Individual species of *Rhizobium* spp. have a distinct host range allowing nodulation of a particular set of legume plants. For example, *Rhizobium leguminosarum* biovar *viciae* nodulates pea and vetch, whereas *B. japonicum* nodulates soybean. At the other extreme, the exceptionally broad host-range *Rhizobium* spp. NGR 234 nodulates 353 legume species representing 122 genera. Differences in both infection processes and organogenic programs are reflected in variations in root nodule morphology but overall there are pronounced developmental similarities as would be expected from a common ancestry (Stougaard, 2000).

Colonization of host cells by *Rhizobium* spp. bacteria involves the progressive remodeling of the plant–microbial interface. Following induction of nodulation genes by legume-derived flavonoid signals, *Rhizobium* spp. secretes Nod-factors (lipochitin oligosaccharides) that cause root hair deformations by perturbing the growth of the plant cell wall. The infection thread arises as a tubular ingrowth bounded by plant cell wall. This serves as a conduit for colonizing bacterial cells that grow and divide in its lumen. The transcellular orientation of thread growth is controlled by the cytoskeleton and is coupled to cell cycle reactivation and cell division processes (Brewina, 2010). *Bradyrhizobium* and *Azorhizobium* spp. grow in the soil as free-living organisms but can

also live as nitrogen-fixing symbionts inside root nodule cells of legume plants (Gage, 2004). In response to *Rhizobium* spp. infection, host cells synthesize several new components that modify the properties of the cell wall and extracellular matrix.

Root nodule extensins are a legume-specific family of hydroxyproline-rich glycoproteins targeted into the lumen of the infection thread. The structural characteristics of these glycoproteins suggest that they may serve to regulate fluid-to-solid transitions in the extracellular matrix. Extensibility of the infection thread is apparently controlled by peroxide-driven protein that cross-links and perhaps modifies the pectic matrix. Endocytosis of rhizobia may occur from unwalled infection droplets into the host cell cytoplasm, which is influenced by physical contact between glycocalyx components of the plant and bacterial membrane surfaces. As endosymbionts, bacteroids remain enclosed within a plant-derived membrane that is topologically equivalent to the plasma membrane. This membrane acquires specialist functions that regulate metabolite exchanges between bacterial cells and the host cytoplasm. Ultimately, however, the fate of the symbiosome is to become a lysosome, causing the eventual senescence of the symbiotic interaction (Brewina, 2010). Bacteria belonging to the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, form indeterminate nodules that arise from root inner and middle cortical cells and grow out from the root via a persistent meristem. During the formation of functional indeterminate nodules, symbiotic bacteria must gain access to the interior of the host root. To get from the outside to the inside, *Rhizobium* spp. grow and divide in tubules called infection threads, which are composite structures derived from the two symbiotic partners (Gage, 2004).

In symbiotic nitrogen fixation, the plant supplies the carbon source for the energy-dependent reduction of dinitrogen and protects the oxygen-sensitive nitrogenase enzyme. All species of genus *Rhizobium* fix nitrogen in symbiosis with legume roots. However, under exceptional conditions, in soils with high soil nitrogen content, legumes do not rely on symbiosis for nitrogen availability (Sprent, 1979). To establish a symbiosis, the bacterial microsymbionts gain access to single plant cells and install themselves in compartments surrounded by a plant membrane. In most symbiotic interactions, a specialized plant organ, the root nodule, develops to provide optimal conditions for the nitrogen-fixing bacteria. Among woody plant species belonging to eight different families, an interaction with the Gram-positive genus *Frankia* leads to the development of actinorhizal root nodules (Stougaard, 2000). In legumes, Gram-negative soil bacteria belonging to the family Rhizobiaceae infect root tissue and induce the formation of the nitrogen fixing nodules. Recent phylogenetic studies based on DNA sequence analysis place all plants involved in rhizobial or actinorhizal symbiosis in the same lineage and suggest that the predisposition for nodulation evolved only once (Stougaard, 2000).

In the most studied legumes, infection occurs via an infection thread that takes the bacteria through the root hair into the root cortex and distributes them to cells, which become the infected cells for the nitrogen-fixing (Gage, 2004). The root zone susceptible to invasion is located behind the root tip where root hairs are still growing and competent for invasion. In response to attached bacteria, root hairs deform and curl setting up a pocket that provides a site for initiating the infection (Udvardi and Poole, 2013). The infection thread is a plant-derived structure originating from plasma membrane

invagination accompanied by external deposition of cell wall material. In advance of the intracellular “inward” progressing thread, root cortical cells differentiate and re-enter the cell cycle.

Cortical cells prepared for infection thread passage appear to be arrested in the G2 phase, whereas cells completing the cycle resume division to establish the nodule primordium (Kiers *et al*., 2003). Later in the process, pattern formation and cell differentiation specify tissue and cell types. The bacteria are endocytosed into a subset of cells where they differentiate into nitrogen-fixing bacteroids surrounded by the peribacteroid membrane of the symbiosome. In the mature functional nodule, peripheral vascular bundles are connected to the root vasculature (Ludwig, 2004). In determinate nodules such as soybean the meristematic activity ceases early, and the nodule grows by expansion giving a spherical shape. All developmental stages from root hair curling to nodule senescence are consequently phased in time. Indeterminate nodules such as pea maintain an active meristem depositing cells that are subsequently infected. This results in a cylindrical shape with the organ formative developmental stages represented along the longitudinal axis. The type of nodule formed is specified by the plant host (Stougaard, 2000).

French beans are nodulated by a host specific *Rhizobium* spp., *Rhizobium leguminosarum* biovar *phaseoli*. The host plant supplies the bacteria with nutrient and shelter in exchange for providing the plant an available source of nutrient. *Rhizobium* are microaerophilic nitrogen fixers within root nodules, thus they are specialized for growth in oxygen limited environments (Ludwig, 2004). Host specificity is regulated by expression of particular

genes, the nod gene (nod D) to the rhizobial plasmid on its host legume (Brockwell *et al*., 1995). Establishment of a symbiotic relationship depends on host-rhizobia detection and recognition, infection, nodule formation and differentiation of rhizobia in nodules. The *Rhizobium* spp. is dependent on the host plant for a continuous supply of compounds synthesized from photosynthesis. The host plant benefits by utilizing the reduced nitrogen and incorporating it into plant tissues.

2.4 French beans

The country of origin of French beans is considered to be South America in particular Peru and Columbia. They were first introduced into Europe by the Spanish conquistadors while in Italy; they were known to be widely grown in vegetable gardens as early as 1569 (www.giorgini.com –accessed on 15th march 2012). From Europe, beans later spread to India, Africa, Indochina, and the rest of the world. French beans are the immature green pods of *Phaseolus vulgaris* grown mainly for export in Kenya (HCDA, 2007). However, local consumption of French beans has been growing gradually. Both large and smallholder farmers grow French beans for fresh consumption and processing. Canning and freezing are the main processing done on French Beans (HCDA, 2011).

Members of the Leguminosae form the largest plant family on earth, with around 18,000 species (Moulin *et al*., 2001). The success of legumes can largely be attributed to their ability to form a nitrogen-fixing symbiosis with rhizobia bacteria, manifested by the development of nodules on the plant roots in which the bacteria fix atmospheric nitrogen, a major contributor to the global nitrogen cycle (HCDA, 2010). Rhizobia described so far

belong exclusively to the subclass of Proteobacteria, where they are distributed in four distinct phylogenetic branches. Although nitrogen-fixing bacteria exist in other proteobacterial subclasses, for example *Herbaspirillum* and *Azoarcus* none has been found to harbor the nod genes essential for establishing rhizobial symbiosis (Moulin *et al*., 2001).

The use of planting certified seeds from reputable seed agents is recommended for planting to ensure they are disease free and well sorted. Before planting, the seed should be dressed with Fernasa-D (combination of Lindane and Thiiram) at the rate of 3 g per kg of seed (NAFIS, 2011). Application of 10 tons farm yard manure (FYM) per hectare is recommended, especially where soils are low in organic matter content. Manure should be applied in planting furrows and worked into soil before planting. Fertilizer requirements at planting are 200 kg D.A.P (Diamonium phosphate) per hectare. The fertilizer should be applied in the planting furrow and mixed thoroughly with the soil before placing the seed (Infonet-biovision.org-beans). Top dressing is done using 100 kg C.A.N (Calcium ammonium Nitrate) per hectare at the first 'three leaves' stage, and another 100 kg per hectare at the onset of flowering. Foliar feed should be applied fortnightly from the forth week after planting to the middle of podding phase (NAFIS, 2011). One hectare takes a total of 50-60 kg of bean seed. Picking begins 7-8 weeks after sowing. Picking is carried out every 2-3 days (Infonet-biovision.org-beans-accessed on 17th January 2012). Regular water supply is essential as moisture affects yields, uniformity and quality. Timely and thorough weeding is absolutely essential. Crops

should not be weeded at flowering time and when the field is wet to avoid flower shedding, spread of diseases and soil compaction (NAFIS, 2011).

2.5 Root-knot nematodes

Root-knot nematodes, *Meloidogyne* species are a growing concern for vegetable producers because chemical nematicides are gradually disappearing from the market and alternative techniques based on agronomic practices to solve the problem are missing (Collange *et al*., 2011). Several studies have already been carried out to address the problem of nematodes, like the use of biointensive integrated pest management (BIPM). However it has less attention. Biointensive integrated pest management (IPM) is defined as 'A systems approach to pest management based on an understanding of pest ecology'. It begins with steps to accurately diagnose the nature and source of pest problems, and then relies on a range of preventive tactics and biological control to keep pest populations within acceptable limits (Reddy, 2013). Reduced-risk pesticides are used if other tactics have not been adequately effective, as a last resort, and with care to minimize risks'.

Biointensive IPM (BIPM) incorporates ecological and economic factors into agricultural system design and decision-making and addresses public concerns about environmental quality and food safety. The benefits of implementing biointensive IPM can include reduced chemical input costs, reduced on-farm and off-farm environmental impacts and more effective and sustainable pest management. Many practices are only partially effective for nematode control as portrayed by Collange *et al*. (2011). In BIPM, several alternative techniques are considered, including sanitation, soil management, organic

amendments, fertilization, biological control and heat-based methods (Reddy, 2013). An ecology-based IPM has the potential of decreasing inputs of fuel, machinery and synthetic chemicals all of which are energy intensive and increasingly costly in terms of financial and environmental impact (Karssen, 2002). Such reductions will benefit the grower and society.

BIPM options may be either proactive or reactive. Cultural control practices are generally considered to be proactive strategies. Proactive practices include crop rotation; resistant crop cultivars including transgenic plants, disease-free seed and plants; crop sanitation; spacing of plants; altering planting dates; mulches among others. The reactive options mean that the grower responds to a situation, such as an economically damaging population of pests, with some type of short-term suppressive action. Reactive methods generally include inundative releases of biological control agents such as *Trichoderma* spp., mechanical and physical controls, botanical pesticides and chemical controls (Reddy, 2013).

Seventeen isolates of the free-living soil fungus *Trichoderma* spp., collected from *Meloidogyne* spp. infested vegetable fields and infected roots in Benin, were screened for their rhizosphere competence and antagonistic potential against *M. incognita*, in greenhouse pot experiments on tomato (Affokpon *et al*., 2011). The five isolates expressing greatest reproductive ability and nematode suppression in pots were further assessed in a typical double-cropping system of tomato and carrot in the field in Benin. All seventeen isolates were re-isolated from both soil and roots at eight weeks after

application, with no apparent crop growth penalty. In pots, a number of isolates provided significant nematode control compared with untreated controls. Field assessment demonstrated significant inhibition of nematode reproduction, suppression of root galling (Kimenju *et al*., 1999), resulting in an increase of tomato yield compared with the non-fungal control treatments. *Trichoderma asperellum* T-16 suppressed second stage juvenile (J_2) densities in roots by up to 80 %; *T. brevicompactum* T-3 suppressed egg production by as much as 86 %. Tomato yields were improved by over 30 % following the application of these biocontrol agents, especially *T. asperellum* T-16. Second stage juvenile (J2) densities were suppressed in treated plots, by as much as 94 % (*T. asperellum* T-12), compared with the non-fungal controls (Affokpon *et al*., 2011).

The root-knot nematodes are obligate plant pathogens. As a result of nematode feeding, large galls or "knots" can form throughout the root system of infected plants. Severe infections result in reduced yields on numerous crops and can also affect consumer acceptance of many products, including vegetables (Karssen, 2002). The name *Meloidogyne* is of Greek origin, meaning "apple-shaped female". Root-knot nematodes are recognized as a major constraint to bean farming in Kenya, causing up to 60 % yield losses in heavily infested fields (Kimenju *et al*., 1999). Root-knot nematodes begin their lives as eggs that rapidly develop into J1 (first-stage juvenile) nematodes.

The J1 stage resides entirely inside the translucent egg case, where it molts into a J2 nematode. The motile J2 stage is the only stage that can initiate infections. Second juvenile stage (J2) attack growing root tips and enter roots intercellularly, behind the root

cap. They move to the area of cell elongation and initiate a feeding site by injecting esophageal gland secretions into root cells. These nematode secretions cause dramatic physiological changes in the parasitized cells, transforming them into giant-cells. As with all nematodes, root-knot nematodes undergo four juvenile stages, where "molting" occurs from one stage to the next (Karssen, 2002). As a result of this process, juvenile root-knot nematodes have little resemblance to adult males and females. In the J4 stage, the progression from juvenile to sedentary globose adult females or to vermiform adult males becomes clearly visible. They emerge as adults from the J4 cuticle. A single female nematode can produce 500 to more than 1000 eggs (Kimenju *et al*., 1999). The length of a root-knot nematode life cycle varies among species but can be as short as two weeks.

Previous study carried out by Singh and Reddy (1981), on the influence of *M. incognita* infestation on *Rhizobium*-nodule formation in French beans showed that root knot nematode infested plants are stunted and generally unthrifty. The root-knot nematodes also cause reduced nodulation in leguminous plants. The nematode infested plants were investigated for other growth parameters and they were found to have reduction in plant height, fresh weight of shoot and root, root length and reduced number of nodule in the root system. *Trichoderma* spp. has been used for the biological control of root-knot nematodes (Sharon *et al*., *2001*). The use of *T. asperellum* in the control of nematodes is therefore important and necessary. Infestation of French beans with nematodes causes a reduction in the plant growth. This is as a result of infestation of the root system by the nematodes. The infested roots develop galls which in turn interfere with the xylem vessels that assist in the uptake of water and mineral salts from the soil to the leaves of

the plant. The functioning of the xylem vessels in the root system is also affected. The phloem vessels are important in the transport of soluble products of photosynthesis from the leaves to the rest of the plant. Such plants will eventually result in reduced yields.

2.6 Role of nitrogen in plants

Nitrogen is the nutrient plants require in the highest amount. Nitrogen availability has a major influence on both yield and product quality. In nature, plants acquire nitrogen by assimilation of nitrate and ammonium or from dinitrogen through association with nitrogen-fixing bacteria (Stougaard, 2000). All plants need nitrogen for healthy growth. Nitrogen is an essential macronutrient needed by all plants inorder for them to grow well and produce high yields It is an important component of many structural, genetic and metabolic compounds in plant cells. It is also one of the basic components of chlorophyll, the compound necessary for photosynthesis (Nasholm *et al*., 2009). Increasing the levels of nitrogen during the vegetative stage can strengthen and support the roots, enabling plants to take in more water and nutrients. This allows a plant to grow more rapidly and produce large amounts of succulent, green foliage, which is especially important in vegetable crops. The plants in turn can generate bigger yields, and a crop that is more resistant to pests, diseases, and other adverse environmental conditions.

A nitrogen-deficient plant is generally stunted because it lacks the nitrogen it requires to manufacture adequate structural and genetic materials (HCDA, 2011). Deficiency symptoms appear first in older leaves which become yellow or pale green due to the lack of chlorophyll, which begin at the tips of the lower leaves and eventually spread

throughout the plant (Stougaard, 2000). In extreme deficiencies, the affected leaves become brownish, wither, and die. Using too much nitrogen, however, can be just as harmful to plants. High levels of nitrogen in plants reduce production of flowers or fruit (www.giorgini.com – accessed on 20th June 2013). As with nitrogen deficiency, the leaves may turn yellow and drop. Too much nitrogen can result in plant burning, which causes them to shrivel and die.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study site was in Mwea area of Kirinyaga County, which is one of the French bean growing areas in Kenya. Previous field trials had been carried out in this region on the use of *T. asperellum* in the control of RKN. As such this site provided a reliable source of *T. asperellum*. Greenhouse and laboratory experiments were carried out at Kenyatta University, Department of Plant and Microbial Sciences. Mwea area is located in Kirinyaga County, in the low midland (LM_5) agro-ecological zone (AEZ), according to Jaetzold and Schmidt (1983). It is a region of medium to high agricultural potential. The average annual rainfall ranges between 640 mm - 2000 mm while the altitude ranges between 760 - 2070 meters above sea level. The soils are fertile due to presence of weathered minerals enriched with volcanic ash. The average soil pH is 6.9 (Jaetzold and Schmidt 1983). Laboratory and greenhouse experiments were carried out in the Department of Plant Sciences Kenyatta University.

3.1.1 Inoculation and culturing of *T. asperellum* and *Rhizobium* spp.

The laminar flow hood was used when inoculation was being carried out in order to ensure an aseptic working area but still allow the culturing of the microbiological organisms. The bench or working surface and the internal surfaces of the hood were sprayed with 70 % ethanol and wiped clean using cotton wool. *Trichoderma asperellum* was inoculated and cultured on potato dextrose agar medium (PDA) which is the preferential medium for culturing of *Trichoderma* spp.

The *Rhizobium leguminosarum* bacteria were inoculated and cultured on yeast mannitol agar (YMA). The YMA was prepared as described by Beck *et al*. (1993), and the composition was as shown in table 3.1.

Table 3.1: Reagents for YMA preparation.

Reagent	**Amount** g/l
Mannitol	10
$MgSO_4.7H_2O$	0.1
K_2HPO_4	0.5
NaCl	0.2
Yeast extract	0.5
Agar	15.0

Reagents and the amounts used in the preparation of YMA. Adopted from Beck *et al*. (1993).

The PDA medium was prepared by weighing 39 grams of PDA and dissolving it in some distilled water as described by Beck *et al*. (1993). The solution was then made up to one liter and sterilized by autoclaving at 121 ^{o}C for 15 minutes. After cooling to about 45 ^{o}C, the medium was poured into sterile petri dishes then left to solidify before inoculation was carried out using a flame sterilized wire loop. The colonies of *T. asperellum* were cultured for seven days at 27 ^{o}C. *Rhizobium leguminosarum* colonies were cultured for five days at the same temperature. Presumptive tests for *Rhizobium* spp. were carried out by culturing the pure cultures of the *Rhizobium* spp. on YMA in Congo red (CR), YMA in bromothymol blue (BTB) as well as Gram stain test. Incubation of the *Rhizobium* spp.

culture on YMA in CR was carried out in the dark while for YMA in BTB the cultures of *Rhizobium* spp. were incubated in presence of light. The growth of the colonies was monitored and their characteristics recorded.

3.2 Preliminary laboratory tests

3.2.1 Isolation and identification of *Rhizobium* spp. isolates

Ten French bean plants which had flowered and nodulated were uprooted from the field research site in Mwea. Systematic random sampling was used to select the ten French beans. Some root nodules were cut to ensure that they had pink active nodules and the roots of the plants cut off and placed in a polythene bag before being transported to the Plant and Microbial Sciences laboratories, Kenyatta University. In the laboratory, the roots were washed under tap water and the nodules detached from the plant. The intact nodules were air dried before being preserved in desiccation vial. The desiccating agent used was silica gel. Cotton wool was placed on the silica gel before placing the nodules to separate the nodules from the silica gel (Somasegaran and Hoben, 1994).

The sterilization, isolation and culturing of *R. leguminosarum* was carried out as per the procedure described by Beck *et al*. (1993). The desiccated nodules were rehydrated in sterile distilled water for one hour. After rehydration, the nodules were surface sterilized in 95 % alcohol for 10 seconds and then transferred to 5 % sodium hypochlorite (NaOCl) solution for 3 minutes. The sterilized nodules were rinsed in seven changes of sterile water and the nodules left in the final rinse. The sterilized nodules were crushed in a sterile test tube with a sterile glass rod in two drops of sterile water. YMA medium was

prepared and sterilized in an autoclave at 121 ^{o}C for 15 minutes. The medium was allowed to cool to 45 ^{o}C after which about 25 ml of the medium was poured into sterile petri dishes. A flame sterilized wire loop was used to scoop a loop full of the suspension which was streaked on the sterilized yeast mannitol agar (YMA) plates and incubated at 27 ^{o}C. The streaking was carried out in a laminar flow.

3.2.2 Presumptive tests of *Rhizobium* spp. isolates

Presumptive tests were carried out on the isolates of *R. leguminosarum* as well as authentication to establish their characteristics. The presumptive tests were Gram stain, growth on YMA containing congo red (CR) and growth on YMA containing Bromthymol blue (BTB) (Somasegaran and Hoben, 1994).

3.2.3 Gram stain test

Gram stain was carried out on the three *Rhizobium* spp. isolates, 3E, 4E and 6E, as described by Beck *et al*. (1993). Observations were made and the results recorded.

3.2.4 Presumptive tests using YMA in CR and YMA in BTB

Each of the three *Rhizobium* spp. isolates was cultured separately on YMA in CR and YMA in BTB. A flame sterilized wire loop was used to scoop a loopful of each of the pure culture which was streaked on a petri dish containing sterilized YMA in CR and another streaking was made on petri dish containing sterilized YMA in BTB. The

cultures that were streaked on YMA in Congo red (CR) were incubated at 27 ^{o}C for four days. The cultures of *Rhizobium* spp. that were streaked in Bromthymol blue were incubated in the open and at 27 ^{o}C. The growth of the cultures was monitored and recorded.

3.2.5 Preservation of isolated *R. leguminosarum* isolates

The culture of *Rhizobium* spp. isolates were preserved in McCartney bottles containing YMA and maintained to minimize loss of viability, genetic variation and contamination. They were preserved on agar slants which were sub cultured every three months to ensure the cultures did not lose viability and effectiveness (Somasegaran and Hoben, (1994). Screw capped McCartney bottles containing slants of YMA were autoclaved for 15 minutes at 121 ^{o}C with the caps loosely fitted. The bottles were left at a slanted position and the agar was left to cool and solidify. Inoculation on the agar slants was carried out using a flame sterilized wire loop. The inoculated slants were incubated at room temperature for three days after which they were stored in the refrigerator at 4 ^{o}C. The culture bottles were labeled to indicate the date and name of the isolates.

3.3 Authentication of *Rhizobium* spp. strains

Authentication was carried out to confirm that the isolates obtained from the French beans in the field were actually *Rhizobium* spp. This was carried out in the greenhouse using modified Leonard jar assemblies and sterilized vermiculite as described by Somasegaran and Hoben (1994). The vermiculite was washed with water and sterilized

by autoclaving at 121 ^{o}C for 15 minutes. The sterilized vermiculite was then allowed to cool before being placed in the upper half of the Leonard jar assembly and the top covered with aluminum foil. Sterilized nitrogen free nutrient was placed in the lower reservoir of the Leonard jar. The whole set up was covered with light proof paper secured with heat resistant tape before being stem sterilized for 15 minutes.

Twenty certified French bean seeds of the star variety, which is grown in the study site, were selected for pre-germination. They were surface sterilized by first being dipped in 95 % alcohol and then surface sterilized using 3 % sodium hypochlorite solution for 3 minutes. The seeds were then rinsed in seven changes of sterile distilled water and left in the final change of sterile water until they were fully imbibed. The sterilized seeds were then transferred using sterile forceps, to the surface of the sterilized 2 % water agar medium in a petri dish for pre-germination.

The water agar medium was prepared by dissolving 2 grams of agar in 98 ml of distilled water. The medium was sterilized by autoclaving at 121 ^{o}C for 15 minutes. The medium was allowed to cool to 45 ^{o}C, after which it was dispensed into sterile petri dish plates and left to solidify. The sterilized French bean seeds were then placed on the plates containing water agar facing downwards in order to obtain straight radicles. The plates were incubated at room temperature for about five days. The radicles were allowed to grow on the medium until they attained a length of about 1 cm.

Using flame-sterilized forceps, a hole was made at the centre of foil covering the vermiculite in the Leonard jar assembly. One pre germinated seedling was transferred to each sterile Leonard jar through the pierced hole (Somasegaran *et al*., 1994). After three days when the cotyledons opened, the seedlings were inoculated with 1 ml of a sterile yeast mannitol broth (YMB) culture containing *Rhizobium* spp. culture which had been grown for three days as shown in plate 3.1.

Plate 3.1: Inoculation of French bean seedlings with the *Rhizobium* spp. isolates.

All tools and equipment that were used in authentication such as the micropipette tips for inoculation with *Rhizobium* spp. were also sterilized by autoclaving at 121 ^{o}C for two hours to safeguard against contamination. The three *Rhizobium* spp. isolates, 3E, 4E and 6E, which were isolated from the French beans in the study site, were inoculated onto the seedlings. Each isolate was used to inoculate two different French bean seedlings. The

effectiveness of each isolate to nodulate the French beans was evaluated. The most effective *Rhizobium* spp. isolate was used for inoculating the French beans in the subsequent greenhouse experiments. The seedlings were allowed to grow for 8 weeks and their growth monitored.

3.3.1 Preparation of Yeast Mannitol Broth (YMB)

The YMB contained mannitol 10.0 grams (g), $MgSO_4.7H_2O$ 0.1 g, K_2HPO_4 0.5 g, NaCl 0.2 g and yeast extract 0.5 g (Beck *et al*., 1993). The ingredients were dissolved in distilled water and the volume adjusted to one liter using a volumetric flask. The YMB was sterilized and cooled to 45 ^oC before being dispensed into McCartney bottles and allowed to cool. A flame sterilized wire loop was used to transfer a loopful of *Rhizobium* spp. culture into the YMB and was allowed to grow. After three days, 1 ml of the *Rhizobium* spp. culture was used for inoculating the French bean seedlings.

3.3.2 Preparation and application of nitrogen free mineral nutrient

The nitrogen free nutrient was prepared as described by Beck *et al.* (1993) as specified for food and forage legumes. Five stock solutions were used to prepare the nitrogen free nutrient as shown in table 3.2. For each liter of full strength solution, 0.5 ml from each of the five stock solutions was used. The stock solutions were measured and placed in a one liter volumetric flask and made up to the one liter mark using distilled water. The solution was then transferred to a conical flask and the mouth covered with aluminum foil. The

solution was sterilized after which it was allowed to cool before being used in the Leonard jar assemblies.

Table 3.2: Stock solutions of nitrogen free nutrient.

Stock solution	**Compound**	**Amount g/l**
1	$CaCl_2.2H_2O$	294.1
2	KH_2PO_4	136.1
3	$MgSO_4.7H_20$	123.3
	K_2SO_4	87.01
	$MgSO_4.H_2O$	0.3338
4	H_3BO_3	0.27
	$ZnSO_4.7H_2O$	0.288
	$NaMoO_2.2H_2O$	0.048
	$CoSO_4.7H_2O$	0.056
5	Fe Citrate	5.4

1 – 5 represent the five stock solutions used in preparing the nitrogen free nutrient.

The nitrogen free nutrient in the reservoirs was replenished with sterile solution of the same when depleted. Growth and nodulation were monitored for 8 weeks after inoculation (Beck *et al*., 1993). Two replicates were set up for each *Rhizobium* spp. isolate. Two replicates for the control were also assembled whereby the French bean seedlings were not inoculated with any *Rhizobium* spp.

3.3.3 Re-isolation of *Rhizobium* spp. isolate from authentication

After harvesting of the French beans from the greenhouse and obtaining the root nodules, the *Rhizobium* spp. was re-isolated from the root nodules as described in section 3.2.1. The re-isolated *Rhizobium* spp. isolate was labeled as *Rhizobium* from the greenhouse (RGH). The process that was followed during the re-isolation of *Rhizobium* spp. isolate was as described by Beck *et al*. (1993). Presumptive tests were carried out on this *Rhizobium* spp. isolate using YMA in CR, YMA in BTB and also Gram stain.

3.4 Establishment of the *T. asperellum* culture

The *T. asperellum* isolate that was used in the research was obtained from a field in Mwea where the same isolate was under evaluation. The *Trichoderma* spp. was embedded on dried sorghum grains where it was preserved and packaged in sealed polythene paper bags. One of the *T. asperellum* embedded sorghum grain was placed at the centre of a sterile petri dish containing sterilized potato dextrose agar (PDA) which is the preferential medium for culturing *Trichoderma* spp. The PDA medium was prepared by weighing 39 grams of PDA and dissolving it in some distilled water. The solution was then made up to one liter and sterilized by autoclaving at 121 ^{o}C for 15 min. The medium was allowed to cool to about 45 ^{o}C before being poured into sterile petri dishes. In each petri dish, about 25 ml of the medium was placed. The medium covered a depth of about 4 mm in the petri dish. Four replicates were set up. The plates were then incubated at 27 ^{o}C for seven days. The growth of the culture was observed and monitored.

3.5 Effect of *T. asperellum* on growth of *R. leguminosarum* *in vitro*

The relationship between *Trichoderma* spp. *and Rhizobium* spp. was established by culturing them on suitable media that supports the growth of both organisms. Initially, *R. leguminosarum* and *T. asperellum* were cultured separately on their preferential media, YMA for *Rhizobium* spp. and PDA for *T. asperellum*. Trials were carried out on YMA and PDA to establish which of the media can support the growth of both organisms. However, YMA was found to be the most suitable medium for culturing both *Trichoderma* spp. and *Rhizobium* spp.

Different techniques were used in order to establish whether the interaction was antagonistic, synergetic or if there was no effect on their growth. The techniques used in plating were spread plating, radial streaking and disc plating. Sterile YMA was placed in sterile petri dish. A 5 mm disc of *T. asperellum* was obtained from a mature culture of *Trichoderma* using a sterilized cork borer and placed in the middle of the PDA medium. Radial streaks were then made using a culture of *Rhizobium* spp. The set up was incubated at 27^{o} C for seven days. The growth of the two cultures was observed and monitored. The interaction was categorized according to the modified five types of interaction grades proposed for fungi interaction by Prince *et al*. (2011) as follows:

1. Mutual intermingling without any macroscopic signs of interaction - Grade 1.
2. Mutual intermingling growth, where the growth of fungus is ceased by growth of opposed organism – Grade 2.
3. Intermingling growth, where the fungus under observation is growing on the opposed organism either above or below - Grade 3.

4. Sight inhibition of both the interacting organisms with narrow delineation line Grade 4.
5. Mutual inhibition of growth at a distance of > 2mm - grade 5.

3.5.1 Dual culture technique

The *T. asperellum* isolates were evaluated against *Rhizobium* spp. by dual culture technique as described by Kucuk and Kivanc (2004). A mycelial disc measuring 5 mm in diameter was taken from the margin of seven day old *Trichoderma* spp. culture using a flame sterilized cork borer. This was inoculated at the centre of a petri dish containing 25 ml of YMA medium. Radial streaking was then carried out on the same plate using a sterile loop. The loop was loaded with 48 hour old culture of *Rhizobium* spp. grown at 27 ^{o}C on YMA. The radial streaking was carried out starting from the centre of the plate where the disc of *T. asperellum* culture had been placed, to the peripheral of the plate. The wire loop was sterilized each time after carrying out one radial streak and before proceeding with the next radial streaking.

The dual culture was incubated at 27 ^{o}C and observed for seven days. The zone of inhibition was measured and recorded on the third, fifth and seventh day. The experimental design was completely randomized design with 4 replicates. The control had no *T. asperellum*; instead a 5 mm sterile disc of agar which had been cut using a flame sterilized cork borer was placed at the centre of the plate containing sterilized YMA. The percentage inhibition of the average radial growth was calculated in relation to growth of the controls (Johnson and Sekhar, 2012) as follows;

$$L = \frac{[C - T] \times 100}{C}$$

L Percentage inhibition of rhizobia radial growth (percentage inhibition).

C Radial growth measurement of the rhizobia in control (radial growth in control).

T Radial growth of rhizobia in the presence of *Trichoderma* (radial growth in the treatment).

3.6 The effect of *T. asperellum* on nodulation in French beans

The greenhouse experiment was set up to investigate the effect of *T. asperellum* on nodulation in French beans under controlled conditions. Other factors that were evaluated included the effects of *T. asperellum* on presence of nematodes, galling index as well as the effects on plant growth parameters. This growth parameters were shoot biomass, root biomass plant biomass and root collar diameter. Soil and sand were sterilized separately over night in an oven at 100 ºC and allowed to cool. The soil and sand were mixed in a ratio of 1:2 respectively. About 2 kg of the sand and soil mixture were placed in each of the 20 pots measuring 16 cm top diameter, and 13 cm bottom diameter and 13 cm in height. Five treatments, designated as T1-T5, were applied to the pots. The treatments were applied to the soil two weeks before planting. Water was applied to the treated soils on a regular basis. The treatments that were applied to the sterilized soil in the pots were as follows;

T1 = Low rate of *T. asperellum* (0.263 g).

T2 = Medium rate of *T. asperellum* (0.396 g).

T3 = High rate of *T. asperellum* (0.527 g).

T4 = Bionematon (standard biological nematicide) (1.538 g).

T5 = Untreated control.

3.6.1 Pre-germination and seedling transfer

The French bean seeds that were planted in the greenhouse were certified seeds of French beans *Phaseolus vulgaris* cv. star which is commonly grown in Mwea and central region of Kenya. Healthy intact French bean seeds without wrinkles were selected. The French beans were first pre-germinated in sterile water agar before transplanting them into the pots. The seeds were first sterilized before placing them in the sterilized water agar medium by placing them in 95 % alcohol for 10 seconds. They were then transferred to 3 % sodium hypochlorite (NaOCl) solution for 3 minutes. The sterilized French bean seeds were rinsed in seven changes of sterile water. They were left in the final rinse of water for one hour in order to imbibe the water. The seeds were then transferred into the petri dish plates containing sterilized water agar medium. The number of French bean seeds per plate was 15 and were spread evenly in each plate. The water agar was prepared by dissolving two grams of agar into 98 ml of distilled water. The solution was sterilized by autoclaving at 121 ^{o}C and 15 Pascal's for 15 minutes. The solution was cooled to 45 ^{o}C before dispensing about 25 ml of the solution into petri dishes. The medium in the petri dishes was left to cool before placing the French bean seeds. The set up was incubated at room temperature for five days and the germination of the seeds monitored. When the radicles of the seedlings attained 1 cm in length, the seedlings were transplanted into the

pots in the greenhouse. A flame sterilized forceps was used to transplant the seedlings into these pots.

Three healthy seedlings were transplanted into each pot ensuring that the seedlings were evenly spread in the pot. A total of 20 pots were used and the dimensions of each pot were 16 cm top diameter and 13 cm as the bottom diameter while the depth of each pot was 13 cm. After transplanting the seedlings, the top of each pot was covered with about 2 cm layer of gravel. The gravel was sterilized overnight in an oven at 100 ^{o}C and allowed to cool before being placed in the pots. The layer was to trap any contaminants and prevent them from reaching the seedlings. The seedlings were watered every two days and left to grow.

3.6.2 Inoculation of French bean seedlings with *Rhizobium* spp. and RKN

The cotyledons opened after three days and thereafter, the seedlings were inoculated with one (1) ml of *Rhizobium* spp. culture. The *Rhizobium* spp. had been cultured in YMB and allowed to grow for three days. The best nodulating *Rhizobium* spp. isolate was used to inoculate the French beans in the greenhouse. The inoculation with *Rhizobium* spp. isolate was carried out five days after transplanting the French beans to the pots. One (1) ml of *Rhizobium* spp. inoculum was applied to the French bean seedlings at the base of the roots using a sterilized micropipette.

One week after inoculation with *Rhizobium* spp. each plant was inoculated with 1500 second juvenile (J2) of root - knot nematodes. The J2s' were isolated from the root galls

of tomato plants and appeared as shown in plate 3.2. The globose females were extracted from the root galls of the infected tomato roots using a mounting needle and placed in some sterile water. The globose females of RKN were isolated and transferred to small beakers partially filled with sterile water.

Plate 3.2: Nematode infested tomato plant roots.

The eggs were incubated overnight at room temperature in order to hatch into the infective juvenile nematodes (J2). The J2s' were inoculated into the soil containing French bean seedlings in the pots. The inoculation was carried out by making three holes at the base of each plant next to the rhizosphere. One globose female contains at least 500

J2s'. One thousand five hundred larvae from three females which were suspended in 10 ml of water were inoculated on each plant. These were inoculated into three holes punched close to the root zone of each seedling (Singh and Reddy, 1981). Repeats of the treatments T1-T4 were carried out 3 weeks after planting. Growth of the French beans was monitored for eight weeks, after which harvesting was done. Growth and nodulation were monitored for 45 days after nematode inoculation before harvesting. Nodulation, galling index and presence of J2s' as well as plant growth parameters were determined.

3.7 Phenological assessment

The French bean plants were harvested from the greenhouse after 45 days from the time of inoculation. The plants were watered well using 400 ml of water one hour before uprooting them to minimize the damage on the roots and also to make it easier to detach the roots from the soil. After one hour the pot together with its contents were turned upside down before lifting the plants carefully from the soil. The roots were carefully removed ensuring that the nodules as well as parts of the roots were not detached and left in the soil. The soil was washed off from the roots.

The factors that were assessed after harvesting were the number of nodules on each plant, galling index and presence of J2s' in the soil, as well as some growth parameters. The growth parameters that were determined included root collar diameter, root biomass and shoot biomass. The root was separated from the shoot by cutting using a scalpel at the point where the stem had been in contact with the soil. The shoots and roots were then placed in separate paper bags and oven dried for three days at 70^{o} C to obtain their

biomass. The nodules were counted and then detached from the roots. The nodules were air dried to obtain their biomass.

3.7.1 Nodule number

Forty five days after inoculation of the French bean seedlings with nematodes, the plants were harvested. The soil was washed off the roots which were then assessed for nodulation. The nodules were separated from the roots by gently pulling them off from the plant. The number of nodules was counted, recorded and the nodule sizes and color recorded (Prevost and Antoun, 2008). Random samples of three nodules were taken and cut to determine the percentage of those that were pink in colour and thus effective in nodulation (Beck *et al*., 1993).

3.7.2 Nodule biomass

The nodules were air dried for two weeks. Weighing was carried out after every two days until a constant dry weight was obtained. The desiccated nodules were preserved in glass vials. The desiccant used was silica gel which was placed at the bottom of the glass vials (Beck *et al*., 1993). Cotton wool was used to separate the nodules from the desiccant.

3.7.3 Extraction of J2s' of RKN from the soil

Root - knot nematodes J2s' were extracted following the method described by Hooper *et al*. (2005). The equipment needed for the extraction of nematodes included a plastic sieve with coarse mesh, a plastic tray or extraction plate and paper napkin, beakers to wash the

extraction into, wash bottle, labels, and weighing scale. Two hundred grams of soil were weighed. A paper napkin was placed in the plastic sieve placed on a plastic plate. A coarse plastic mesh sieve was placed on a plastic tray and its bottom and sides covered with a layer of a serviette tissue paper. Two hundred grams of finely crumbled soil was spread out over the serviette paper as shown in plate 3.3. Clean water was carefully added down the sides of the collecting tray until the soil layer was wet. The plastic tray and the sieves were labeled to indicate the type of treatment from which the soil sample had been obtained. The set up were left overnight for 24 hours. The J2s' were harvested by pouring the suspension collected on the tray over a sieve size of 38 µm. The J2 nematodes were backwashed into a small beaker with 20 - 30 ml of water from a wash bottle. The J2 nematodes were observed and enumerated using a compound stereo-microscope, and their number recorded.

Plate 3.3: Extraction of J2s' from the soil using modified Baermann's method.

After 24 hours the sieve was lifted to drain the water into the extraction plate. The soil sample was disposed off from the sieve. The water from the plate was poured into a labeled beaker. Water in a wash bottle was used to rinse the plate and this water added into the beaker. The volume of the water collected was reduced by pouring through a 38 µm Baermann's sieve into a small beaker and concentrated to about 25 ml. The sieve was backwashed to ensure that no J2 nematodes were lost.

3.7.4 Enumeration of root - knot nematodes J2's

The set up was left to settle for about an hour. One (1) ml of the water containing the J2 nematode sample was drawn using a pipette, placed on a counting chamber and observed under a compound stereo-microscope with under stage lighting. The nematodes were

enumerated and recorded as described in section 3.7.3. The nematodes were then placed in screw capped McCartney bottles and preserved in the refrigerator at 4 ^{o}C.

3.7.5 Effect of *T. asperellum* on galling of French beans

The French bean plants were carefully uprooted and assessed for presence of root galls caused by infection of the roots by the nematodes. The galling index was assessed by counting the number of galls observed on the roots and was evaluated and recorded on a scale of 0 to 10 (Coyne *et al*., 2007) as follows;

0	No knots on roots.
1	Few small knots difficult to find.
2	Small knots only but clearly visible. Main roots clean (without galls).
3	Larger knots visible. Main roots clean.
4	Larger knots predominate but main roots clean.
5	Fifty percent (50 %) of roots affected, knotting on some main roots; reduced root system.
6	Knotting on main roots majority of main roots knotted.
8	All main roots, including tap root, knotted. Few clean roots visible.
9	All roots severely knotted, plant usually dying.
10	All roots severely knotted. No root system, plant usually dead.

3.7.6 Effect of *T. asperellum* on root collar diameter of French beans

The root collar diameter was measured at the base of the plant where the soil level had touched the stem. This was carried out using a caliper and a ruler marked in millimeters. The root diameter was recorded and analyzed.

3.7.7 Effect of *T. asperellum* on root biomass of French beans

The roots were separated from the shoot by cutting using a scalpel at the point where the stem had been in contact with the soil. The roots were placed in a labeled paper bag and oven dried at 70 $^{\circ}$C for 3 days to obtain a constant dry weight. The biomass of the roots of each plant was measured and recorded using the JT series electronic balance model JT 202 N.

3.7.8 Effect of *T. asperellum* on shoot biomass of French beans

The shoots were separated from the roots by cutting using a scalpel and placed in paper bags. Their biomass was obtained as described in section 3.7.7. The dry weight was recorded using the JT series electronic balance model JT 202 N and analyzed.

3.8 Experimental design

Five treatments were applied in the pots and these were replicated four times. The total number of pots that were used was twenty. Control experiments had no treatment applied to verify the results obtained by comparing nodulation in instances where no treatment was applied. The experimental design used was completely randomized design (CRD).

3.9 Data analysis

The data collected was summarized using descriptive statistics and then analyzed using qualitative and quantitative statistical methods. Statistical analysis was carried out to determine whether the nodulation and galling differences observed were due to the applied treatments. Analysis of variance (ANOVA) was carried out using SPSS statistical software version 16. The differences between means were separated using Tukey's test at 5 % probability level.

CHAPTER FOUR

RESULTS

4.1 Inoculation and culturing of *T. asperellum* and *R. leguminosarum*

The inoculated cultures of *T. asperellum* and *Rhizobium* spp. had the typical characteristic expected of each of them as described in the preliminary laboratory tests in table 4.1. The use of the laminar flow hood was important in eliminating contaminants since no contaminants were observed.

4.2 Preliminary laboratory tests

4.2.1 Isolation and identification of *Rhizobium* spp. isolates

Colonies started to appear on the second day after inoculation and by the fifth day they had fully matured. The first isolates obtained from the root nodules were a mixture of colonies from which the types typical of *Rhizobium* were sub - cultured. A total of six pure isolates were obtained. For the purpose of this study, the isolates were identified as 1E, 2E, 3E, 4E, 5E, and 6E. After studying their morphological characteristics, the isolates were categorized into three groups, 3E, 4E and 6E, based on their morphological similarities as shown in table 4.1. These characteristics were: - diameter of the colonies, type of margin, elevation, colour and texture of the colony. Presumptive tests were carried out on the isolates 3E, 4E and 6E.

Table 4.1: Morphological characteristics of *Rhizobium* spp. isolates at day 5.

***Rhizobium* Isolates**	**Colony diameter (mm)**	**Margin Type**	**Elevation**	**Colony Colour**	**Colony Texture**
1E	3	Smooth	Convex	Milky white	Mucoid and firm
2E	3	Smooth	Convex	Milky white	Mucoid and firm
3E	3	Smooth	Convex	Milky white	Mucoid and firm
4E	1	Smooth	Convex	Creamy white	Mucoid and firm
5E	1	Smooth	Convex	Creamy white	Mucoid and firm
6E	3	Smooth	Convex	Creamy yellow	Mucoid and firm

1E, 2E, 3E, 4E, 5E, and 6E represent the six isolates of *Rhizobium* species.

After further isolation and sub-culturing of the pure colonies of each isolate, the isolates 1E, 2E and 3E were found to have similar morphological characteristics and they were grouped together and identified as isolate 3E. Isolate 4E and 5E were found to have similar morphological characteristics and were therefore grouped together and identified as isolate 4E. Isolate 6E remained on its own group due to its unique characteristics in respect to colony colour and diameter.

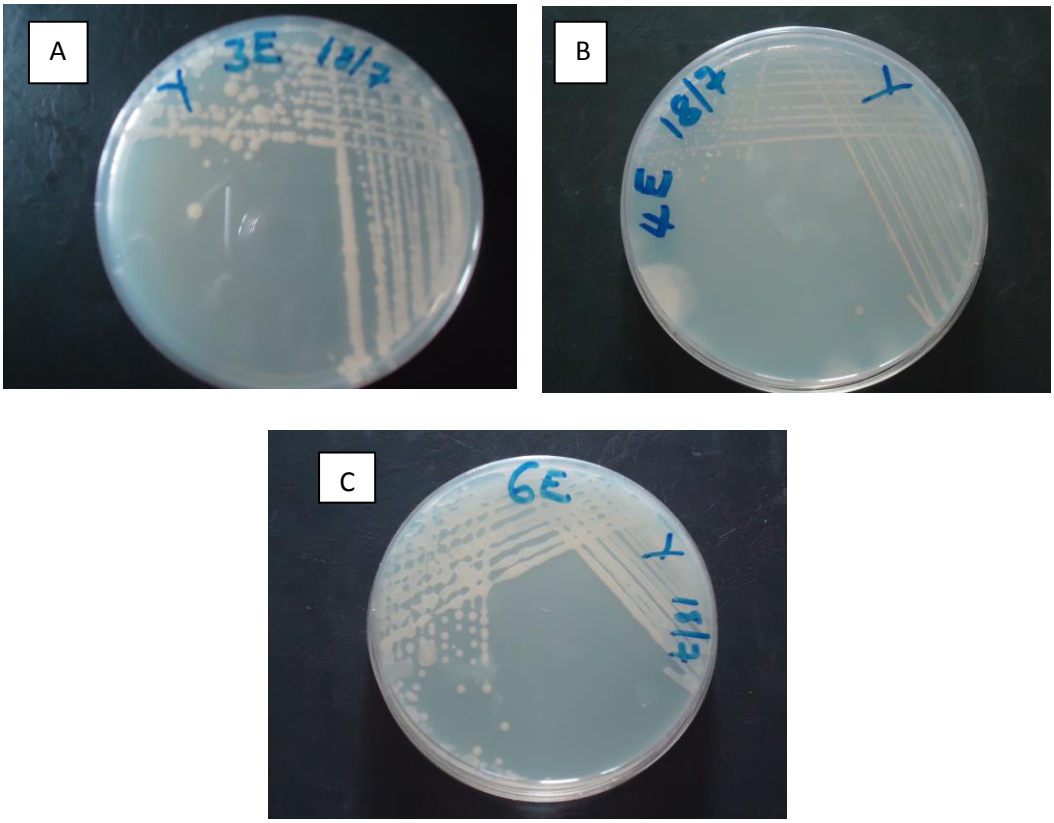

Plate 4.1: *Rhizobium* spp. isolates cultured on YMA. A,3E; B,4E and C,6E.

The morphological characteristics of the *Rhizobium* spp. isolates were determined on the fifth day. The isolates 3E, 4E and 6E when they were grown in YMA appeared as shown in plate 4.1. Their morphological characteristics were as shown in table 4.1. In all the isolates the colonies had fully established by the fifth day.

4.2.2 Presumptive tests on *Rhizobium* spp. isolates

Six isolates of *Rhizobium* spp. were obtained and labeled as 1E – 6E. The isolates were further classified into three isolates, 3E, 4E and 6E based on similarities in their

morphological characteristics. The Gram stain results were as shown in table 4.2 while the results on the presumptive tests were as shown in table 4.3.

4.2.3 Gram stain reaction for the *Rhizobium* spp. isolates

The three isolates of *Rhizobium* spp., that is 3E, 4E, and 6E, retained a pink colour after the Gram stain test was carried out. The isolates were Gram negative and rod shaped. The results from the gram stain were as shown in table 4.2.

Table 4.2: Gram stain results on *Rhizobium* spp. isolates from French beans.

Isolate	Gram stain
3E	(-ve)
4E	(-ve)
6E	(-ve)

3E, 4E and 6E: The different *Rhizobium* spp. isolates; (-ve), Gram negative reaction.

4.2.4 Presumptive tests using YMA in CR and in BTB

The three *Rhizobium* spp. colonies were milky white to translucent and showed little or no absorption of the Congo red dye as shown in table 4.3. When the *Rhizobium* spp. isolate 3E was cultured in YMA in BTB the media turned yellow in colour. The isolate 3E showed no absorption of the Congo red dye when cultured in YMA in CR. Presumptive tests were carried out on the *Rhizobium* spp. isolate 4E turned the medium to a yellow colour when cultured in YMA in BTB and showed no absorption of the Congo

red dye. The presumptive tests carried out on *Rhizobium* spp. isolate 6E had similar results too, whereby the culture grown in YMA in BTB turned the medium yellow in colour. When the isolate 6E was cultured in YMA in CR, the isolate showed no absorption of the Congo red dye as shown in plate 4.2.

Table 4.3: Presumptive tests on *Rhizobium* spp. isolates.

Isolates	**Colour in BTB**	**Colour in CR**
3E	Y	X
4E	Y	X
6E	Y	X

3E, 4E and 6E: The different *Rhizobium* spp. isolates; Y, Yellow colour; X, No absorption of CR.

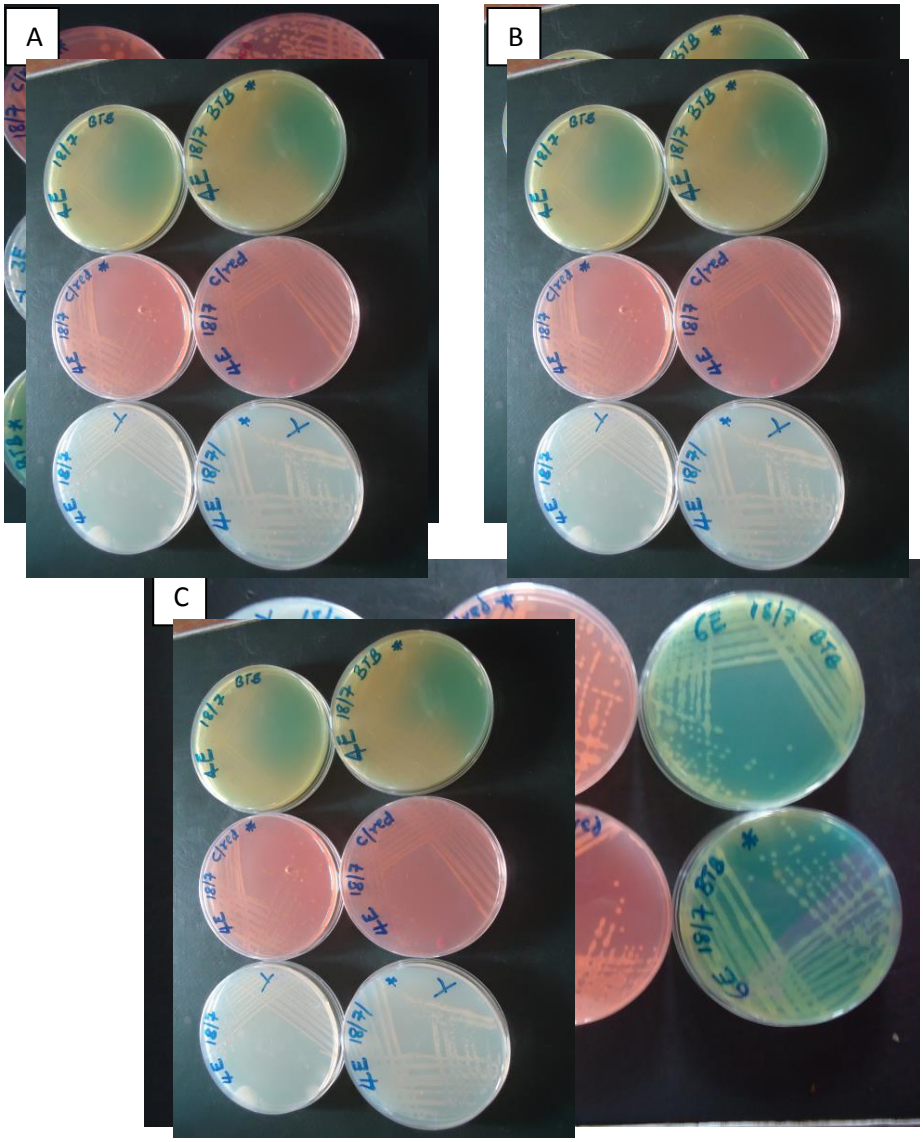

Plate 4.2: Presumptive tests with YMA in BTB and in CR on the *Rhizobium* isolates A, isolate 3E; B, isolate 4E; C, isolate 6E.

4.3 Authentication of the *Rhizobium* spp. isolates

Authentication was ascertained by the ability of the isolated *Rhizobium* spp. to form nodules on French beans under sterile conditions free of *Rhizobium* spp. All the three isolates caused nodulation in the French beans as shown in table 4.4.

Table 4.4: Nodulation from authentication of *Rhizobium* spp. isolates.

***Rhizobium* spp. isolate**	**Replicate 1**	**Replicate 2**
3E	(+ve)	(+ve)
4E	(+ve)	(+ve)
6E	(+ve)	(+ve)
Control	(-ve)	(-ve)

3E, 4E and 6E-*Rhizobium* spp. isolates; (+ve**)** - Nodulation present; (-) - Nodulation absent.

The best nodulating *Rhizobium* isolate was found to be 6E. It formed larger and more nodules compared to the isolates 3E and 4E isolates. The nodule number was significantly different (p=0.00) in which isolate 6E had the highest mean of 15.00 as shown in table 4.5. This was followed by the isolate 4E and 3E with means of 9.00 and 8.50 respectively. The means for isolate 3E and 4E were not significantly different. Isolate 6E was therefore used for inoculation of the French beans in the greenhouse experiments.

Table 4.5: Nodule number from authentication with *Rhizobium* spp. isolates.

***Rhizobium* Isolate**	**Mean ±SE**
3E	8.50 ± 0.50^{a}
4E	9.00 ± 0.50^{a}
6E	15.00 ± 1.00^{b}

Data are the mean ± standard error (SE) of the number of nodules obtained from 2 plants (n=2) from each *Rhizobium* spp. isolate. Means within a column followed by the same letter are not significantly different by Tukey's test at 5 % level.

4.3.1 Growth of *Rhizobium* spp. in YMB

The YMB used to culture *Rhizobium* spp. became increasingly turbid from the second day when the *Rhizobium* spp. colony started to grow. The YMB reached maximum turbidity on day five when the *Rhizobium* spp. culture was fully mature.

4.3.2 Application of nitrogen free mineral nutrient

The seedlings that were treated with nitrogen free mineral nutrient grew into healthy plants on which various phenological assessments were carried out.

4.3.3 Re-Isolation of *Rhizobium* spp. from authentication

The *Rhizobium* spp. isolate RGH had characteristics that were similar to the 6E isolate that had been used to inoculate the French beans in the greenhouse. The characteristics of the isolate RGH on day 5 were as follows; the colonies were 3 mm in diameter, cream

yellow in colour and had smooth margin (Plate 4.3). The elevation was convex with a mucoid and firm texture. The results of the presumptive tests on the isolate RGH were as shown in table 4.6.

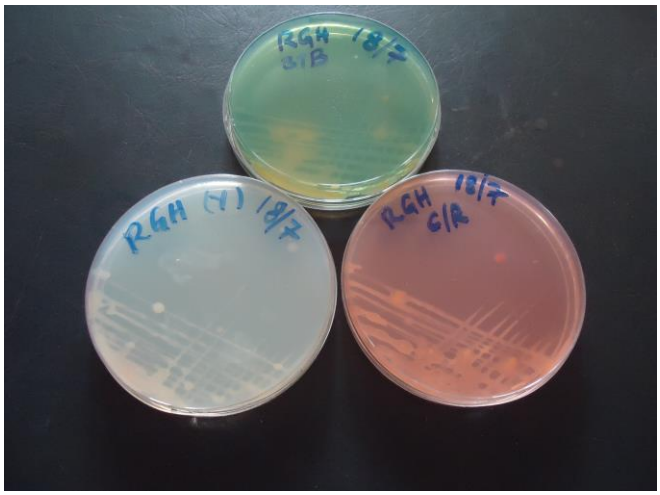

Plate 4.3: Presumptive tests on RGH *Rhizobium* spp. isolate.

Table 4.6: Presumptive tests on *Rhizobium* spp. isolate RGH.

Presumptive tests	**Results**
Gram stain	(-ve)
YMA in BTB	Y
YMA in CR	X

(-ve): Negative; Y, Yellow; X, No absorption of Congo red dye.

4.4 Establishment of *T. asperellum* culture

As the *T. asperellum* colony grew in PDA media, it formed concentric rings and changed colour from white to green as it matured and sporulated as shown in plate 4.4. The conidia formed within seven days in compact tufts of white and green shades. A yellow pigment was secreted into the agar, due to the breakdown of PDA.

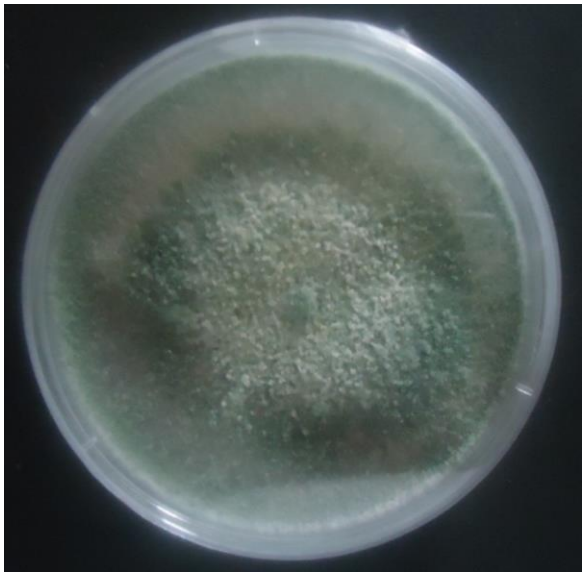

Plate 4.4: *T. asperellum* culture at day 7.

4.5 Effect of *T. asperellum* on growth of *R. leguminosarum in vitro*

Plate 4.5 shows the inhibitory effects exhibited by *T. asperellum* on the growth of *Rhizobium* spp. culture. The radial growth of *Rhizobium* spp. in presence of *T. asperellum* was inhibited as shown in plate 4.5A compared to the radial growth of *Rhizobium* spp. in the control (Plate 4.5B). YMA was found to be the best medium for use in the dual culture technique since it supported growth of *T. asperellum* and is also the preferential medium for culturing *Rhizobium* spp.

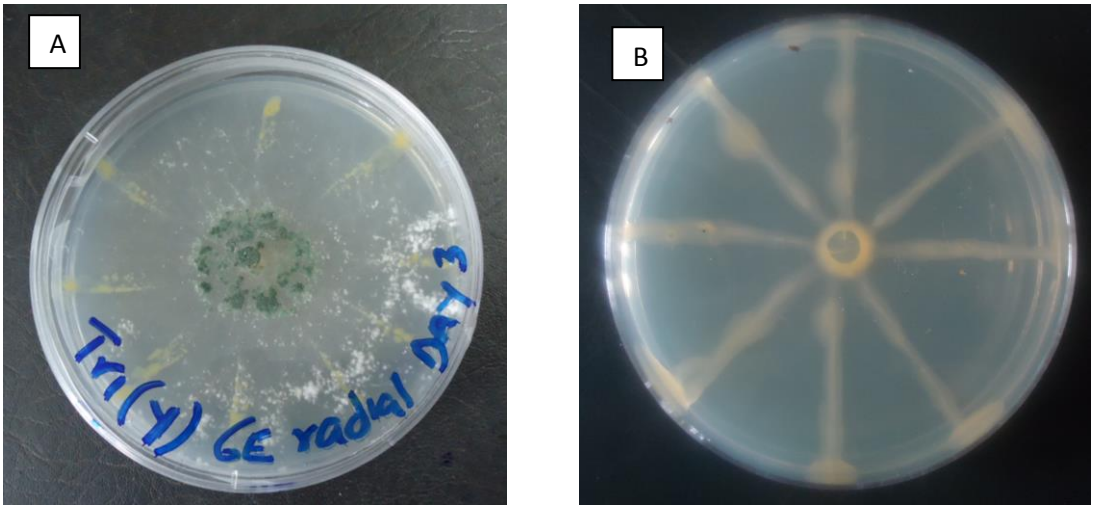

Plate 4.5: Dual culture technique of *T. asperellum* and *Rhizobium* spp. on day 3.
A, Radial growth of *Rhizobium* spp. on YMA in presence of *T. asperellum*; B, Radial growth of *Rhizobium* spp. in the control.

4.5.1 Dual culture technique

On the third day, the *Rhizobium* spp. and *T. asperellum* cultures were well established. The percentage inhibition of *Rhizobium* spp. by *T. asperellum* was as shown in table 4.7. *Trichoderma* spp. was found to inhibit the growth of *Rhizobium* spp. between 49.7 % to 100 %. On the fifth day when *Rhizobium* spp. culture was expected to be fully mature, the average inhibition of *Rhizobium* spp. was 72.35 % and only a little amount of it could be seen on the petri plate. On the seventh day no *Rhizobium* spp. cultures were visible. The concentric rings of *T. asperellum* changed to a green colour by the seventh day, when the *T. asperellum* culture became fully mature and sporulated.

Table 4.7: Percentage inhibition of *Rhizobium* spp. at day 3, 5 and 7.

Day and replicates	**Radial growth in control (C) (mm)**	**Average. radial growth in test petri plate (T) (mm)**	**Percentage (%) inhibition (L)**
Day 3	60	29.7	50.5
Day 3	60	30.2	49.7
Day 3	60	29.7	50.5
Day 3	60	30.1	49.8
Day 5	60	16.0	73.0
Day 5	60	16.7	72.2
Day 5	60	16.4	72.7
Day 5	60	17.1	71.5
Day 7	60	0.0	100
Day 7	60	0.0	100
Day 7	60	0.0	100
Day 7	60	0.0	100

Data are the average radial growth (mm) of *Rhizobium* spp. in the control and in the dual culture technique at day 3, 5 and 7 of four replicates (n=4).

4.5.2 Mean of Radial growth of *Rhizobium* spp. in presence of *T. asperellum*

The means for the radial growth of *Rhizobium* spp. in presence of *T. asperellum* were highly significant (p=0.00). The measurements were taken on day 3, 5 and day 7 and were all found to be significantly different as shown in table 4.8. The growth on day 3

was the highest with a mean growth of 29.93 mm followed by the growth on day 5 with a mean of 16.53 mm. There was no rhizobial growth visible on day 7.

Table 4.8: Mean of Radial growth of *Rhizobium* spp. in presence of *T. asperellum.*

Measurement day	Mean ± SE
Day 3	29.93 ± 0.13^{c}
Day 5	16.53 ± 0.21^{b}
Day 7	0.00 ± 0.00^{a}

Data are the mean ± standard error (SE) of the radial growth of *Rhizobium* spp. in presence of *T. asperellum* obtained from four replicates (n=4) which were measured on day 3, 5 and day 7. Means within the column followed by the same letter are not significantly different according to Tukey's test at 5 % level.

4.5.3 Mean of Percentage inhibition of *Rhizobium* spp.

The means for percentage inhibition were highly significant (p=0.00). Radial growth on day 7 gave the highest inhibition mean of 100 %. The least inhibition mean was on day 3 at 50.13 % while on day 5 the mean inhibition was 72.35 % as shown in table 4.9.

Table 4.9: Mean of Percentage inhibition of *Rhizobium* spp. by *T. asperellum.*

Measurement day	Mean ± SE
Day 3	50.13 ± 0.22^{a}
Day 5	72.35 ± 0.33^{b}
Day 7	100 ± 0.00^{c}

Data are the means ± standard error (SE) of the % inhibition obtained from four replicates (n=4) which were measured on day 3, 5 and day 7. Means within the column followed by the same letter are not significantly different by Tukey's test at 5 % level.

4.6 The effect of *T. asperellum* on nodulation in French beans

Trichoderma asperellum influenced various phenological characteristics that were assessed. The mean number of nodules from the different treatments differed significantly as shown in section 4.7.1. The seedlings got well established in the pots and grew into healthy plants as in plate 4.6.

Plate 4.6: French beans growing in the greenhouse.

4.7 Phenological assessments

The nodules were found to be pink in colour when cut. The means of the number of root galls differed significantly in the different treatments. The structure of the root galls and the nodules were as in plate 4.7.

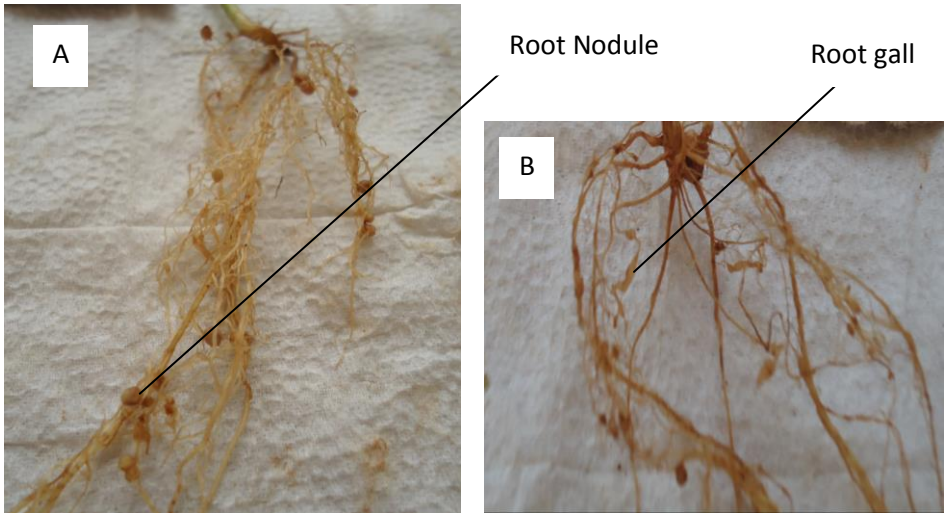

Plate 4.7: Root nodules and root galls on French beans. A , Root nodules on the French bean from the greenhouse; B, Root galls on the French bean from the greenhouse.

4.7.1 Effect of *T. asperellum* on nodule number

The means of nodule number were highly significant (p = 0.00) indicating that the treatments had great influence on the number of nodules formed in the French beans. When the means were separated by Tukey's test, they were found to be different as shown in the table 4.10. The means for low level *T. asperellum* and bionematon were not significantly different. The control gave the highest mean for nodule number of 19.67 followed by treatment with low level *T. asperellum* with a mean of 17.17. Treatment with high level *T. asperellum* gave the lowest mean of 8.08 which was not significantly different with application of *T. asperellum* at medium level which had a mean of 9.50.

Table 4.10: Effect of different treatments of *T. asperellum* on the nodule number.

Treatments	Mean ± SE
Low level Trich	17.17 ± 1.11^{bc}
Mid level Trich	9.50 ± 0.60^{a}
High level Trich	8.08 ± 0.91^{a}
Bionematon	14.58 ± 1.13^{b}
Control	19.67 ± 1.27^{c}

Data are the mean ± standard error (SE) of the number of nodules obtained from 12 plants (n=12) in each treatment. Means within the column followed by the same letter are not significantly different by Tukey's test at 5 % level. Treatments: Low level Trich -Application of *T. asperellum* at low level (0.263 g); Mid level Trich - *T. asperellum* application at medium level (0.396 g); High level Trich - Application of *T. asperellum* at high level (0.527 g); Control - No treatment applied; Bionematon - Treatment with a standard biological nematicide (1.58 g).

4.7.2 Effect of *Trichoderma* spp. on nodule biomass

The means of nodule biomass were not significantly different ($p \geq 0.05$) as shown in table 4.11. All the means from the different treatments were the same ($p = 0.13$). Control had a mean of 0.013, while treatment with bionematon and medium level *T. asperellum* had the same mean of 0.011. Treatment with low level *T. asperellum* had a mean of 0.010 while treatment with high level *T. asperellum* had a mean of 0.007.

Table 4.11: Effect of different treatments of *T. asperellum* on nodule biomass.

Treatments	Mean ± SE
Low level Trich	0.010 ± 0.002^{a}
Mid level Trich	0.011 ± 0.001^{a}
High level Trich	0.007 ± 0.001^{a}
Bionematon	0.011 ± 0.001^{a}
Control	0.013 ± 0.002^{a}

Data are the mean ± standard error (SE) of the number of nodules obtained from 12 plants (n=12) in each treatment. Means within the column followed by the same letter are not significantly different by Tukey's test at 5 % level. Treatments: Low level Trich -Application of *T. asperellum* at low level (0.263 g); Mid level Trich - *T. asperellum* application at medium level (0.396 g); High level Trich - Application of *T. asperellum* at high level (0.527 g); Control - No treatment applied; Bionematon - Treatment with a standard biological nematicide (1.58 g).

4.7.3 Extraction of J2's from the soil

Plate 4.8 shows one of the J2's that was extracted from the soil and observed under the compound stereo microscope.

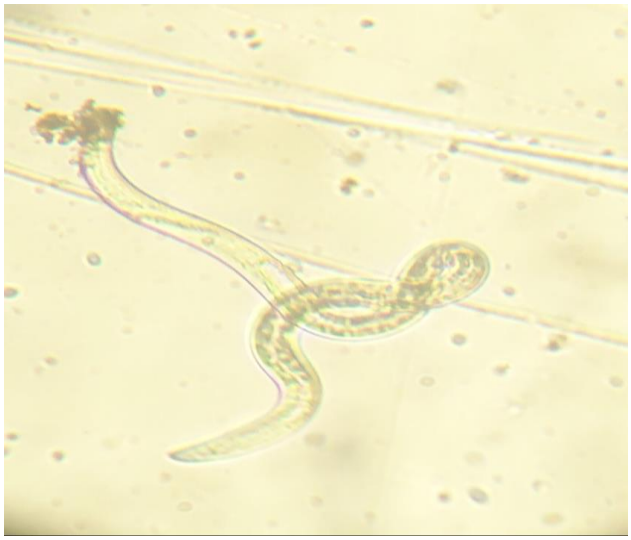

Plate 4.8: J2 of RKN from the soil in the greenhouse.

4.7.4 Enumeration of J2 of root-knot nematodes from the soil

The means of nematode number were highly significant (p = 0.00), indicating that the treatments had great influence on the number of nematodes found in the French beans. The treatments with high level *T. asperellum*, medium level *T. asperellum* and treatment with bionematon had means of 2.00, 2.25 and 2.75 respectively. Their means were not significantly different (p = 0.05) and were the lowest. The treatment with low levels of *T. asperellum* and the control had means that were significantly different. The control had the highest mean for the number of nematodes at 6.75 while treatment with low levels of *T. asperellum* had the mean of 4.00 as shown in table 4.12.

Table 4.12: Enumeration of nematode number from different treatments.

Treatments	Mean ± SE
Low level Trich	4.00 ± 0.37^{b}
Mid level Trich	2.25 ± 0.13^{a}
High level Trich	2.00 ± 0.21^{a}
Bionematon	2.75 ± 0.25^{a}
Control	6.75 ± 0.25^{c}

Data are the mean ± standard error (SE) of the number of nodules obtained from 12 plants (n=12) in each treatment. Means within the column followed by the same letter are not significantly different by Tukey's test at 5 % level. Treatments: Low level Trich -Application of *T. asperellum* at low level (0.263 g); Mid level Trich - *T. asperellum* application at medium level (0.396 g); High level Trich - Application of *T. asperellum* at high level (0.527 g); Control - No treatment applied; Bionematon - Treatment with a standard biological nematicide (1.58 g).

4.7.5 Effect of *T. asperellum* on galling index

The means of galling index, an indication of root infection by RKN were highly significant (p = 0.00) in the French beans. The control had the highest mean for galling index at 5.91 and this mean was significantly different. The means for the other treatments were not statistically different (p = 0.05) as shown in table 4.13. However the means for these treatments were as follows: low level *T. asperellum* 2.33, Bionematon 1.50, application of high level *T. asperellum* 1.42 and treatment with medium level *T. asperellum* 1.08.

Table 4.13: Effect of different treatments of *T. asperellum* on galling index.

Treatment	Means ± SE
Low level Trich	2.33 ± 0.038^{a}
Mid level Trich	1.08 ± 0.34^{a}
High level Trich	1.42 ± 0.29^{a}
Bionematon	1.50 ± 0.31^{a}
Control	5.91 ± 0.51^{b}

Data are the mean ± standard error (SE) of the number of nodules obtained from 12 plants (n=12) in each treatment. Means within the column followed by the same letter are not significantly different by Tukey's test at 5 % level. Treatments: Low level Trich -Application of *T. asperellum* at low level (0.263 g); Mid level Trich - *T. asperellum* application at medium level (0.396 g); High level Trich - Application of *T. asperellum* at high level (0.527 g); Control - No treatment applied; Bionematon - Treatment with a standard biological nematicide (1.58 g).

4.7.6 Effect of *T. asperellum* on root collar diameter

The means of root collar diameter were not significantly different ($p = 0.71$). The means were also not statistically different. However the treatment with bionematon had a mean of 2.75 followed by treatment with high level *T. asperellum* at 2.67. Treatment with medium and low level *T. asperellum* had the same mean of 2.58. The control had a mean of 2.54 as shown in table 4.14.

Table 4.14: Effect of different treatments of *T. asperellum* on root collar diameter.

Treatments	Means ± SE
Low level Trich	2.58 ± 0.10^{a}
Mid level Trich	2.58 ± 0.12^{a}
High level Trich	2.67 ± 0.11^{a}
Bionematon	2.75 ± 0.09^{a}
Control	2.54 ± 0.13^{a}

Data are the mean ± standard error (SE) of the number of nodules obtained from 12 plants (n=12) in each treatment. Means within the column followed by the same letter are not significantly different by Tukey's test at 5 % level. Treatments: Low level Trich -Application of *T. asperellum* at low level (0.263 g); Mid level Trich - *T. asperellum* application at medium level (0.396 g); High level Trich - Application of *T. asperellum* at high level (0.527 g); Control - No treatment applied; Bionematon - Treatment with a standard biological nematicide (1.58 g).

4.7.7 Effect of *T. asperellum* on root biomass

The means of root biomass were not significant (p = 0.21) in the French beans. The means were also not statistically different as shown in table 4.15. The treatment with high level *T. asperellum* had a mean of 0.08 followed by treatment with low level *T. asperellum* with a mean of 0.06. The control and treatment with medium levels *T. asperellum* had the same mean of 0.05. Treatment with bionematon had a mean of 0.04.

Table 4.15: Effect of different treatments of *T. asperellum* on root biomass.

Treatments	Means ± SE
Low level Trich	0.06 ± 0.01^{a}
Mid level Trich	0.05 ± 0.01^{a}
High level Trich	0.08 ± 0.02^{a}
Bionematon	0.04 ± 0.01^{a}
Control	0.05 ± 0.01^{a}

Data are the mean ± standard error (SE) of the number of nodules obtained from 12 plants (n=12) in each treatment. Means within the column followed by the same letter are not significantly different by Tukey's test at 5 % level. Treatments: Low level Trich -Application of *T. asperellum* at low level (0.263 g); Mid level Trich - *T. asperellum* application at medium level (0.396 g); High level Trich - Application of *T. asperellum* at high level (0.527 g); Control - No treatment applied; Bionematon - Treatment with a standard biological nematicide (1.58 g).

4.7.8 Effect of *T. asperellum* on shoot biomass

The means of shoot biomass were not significantly different ($p = 0.08$) as shown in table 4.16. However the treatment with high level *T. asperellum* had the same mean with the control of 0.38. This was followed by the mean for application of low levels of *T. asperellum* at 0.37. Treatment with medium level *T. asperellum* had a mean of 0.31 while bionematon had a mean of 0.22.

Table 4.16: Effect of different treatments of *T. asperellum* on shoot biomass.

Treatments	Mean ± SE
Low level Trich	0.37 ± 0.04^{a}
Mid level Trich	0.31 ± 0.04^{a}
High level Trich	0.38 ± 0.06^{a}
Bionematon	0.22 ± 0.03^{a}
Control	0.38 ± 0.04^{a}

Data are the mean ± standard error (SE) of the number of nodules obtained from 12 plants (n=12) in each treatment. Means within the column followed by the same letter are not significantly different by Tukey's test at 5 % level. Treatments: Low level Trich - Application of *T. asperellum* at low level (0.263 g); Mid level Trich - *T. asperellum* application at medium level (0.396 g); High level Trich - Application of *T. asperellum* at high level (0.527 g); Control - No treatment applied; Bionematon - Treatment with a standard biological nematicide (1.58 g).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Three major Rhizobium isolates were found to infect French beans in Mwea area of Kirinyaga County, Kenya. All the *Rhizobium* spp. isolates had the expected typical morphological characteristics of the genus. When the isolates were grown on YMA in BTB, they turned bromothymol blue yellow, showing they were acid producing bacteria. Bromthymol blue is a pH indicator which turns the green YMA to yellow colour for acid producing bacteria. This assists in distinguishing between *Bradyrhizobium* and *Rhizobium* spp. which are in the same genus but nodulate different crops. *Rhizobium* spp. is a fast grower since they grow within the first three days unlike *Bradyrhizobium* which is a slow grower and turns BTB blue (Beck *et al*., 1993). The *Rhizobium* spp. isolates were cultured in YMA in Congo red which suppresses growth of actinomycetes as well as assisting in recognition of *Rhizobium* spp. (Somasegaran and Hoben, 1994).

When they were viewed under the compound microscope, *Rhizobium* spp. isolates were found to be rod shaped gram negative bacteria, whose cell wall is generally thinner than those of gram positive bacteria and have higher lipid content than the gram positive bacteria (Beck *et al*., 1993). All the isolates of *Rhizobium* spp. were found to be authentic since they all caused nodulation on the French bean plants. However isolate 6E was found to be better than others in nodulation since the mean for the number of nodules was found to be significantly different. The nitrogen free nutrient medium that was used during authentication was able to supply both the macro and micro nutrients required by

the French beans except for nitrogen. The nutrients that were supplied by the medium were phosphates, sulphates, calcium, magnesium, sodium, molybdenum, cobalt, boron, zinc and iron.

Trichoderma asperellum was found to inhibit the growth of *Rhizobium* spp. *Trichoderma* spp. has been found to be mycoparasitic on other organisms which it interacts with (Xiaoxue *et al*., 2013). YMA was found to be the best medium for demonstrating the type of interaction between *Trichoderma* spp. and *Rhizobium* spp. YMA is the preferential medium for culturing *Rhizobium* spp. while PDA is the preferential medium for the culture of fungi such as *T. asperellum* (Harman, 2006). However, YMA was also found to support the growth of *T. asperellum*. The radial method was found to be the most suitable for investigating the type of interaction between *Rhizobium* spp. and *T. asperellum* under the dual culture technique described by Kucuk and Kivanc (2004).

The other methods that had been tried out include disc plating with the two microorganisms on opposite sides of the petri plate (Benitez *et al*., 2004). With this method, it was not possible to tell the type of interaction between *Trichoderma* spp. and *Rhizobium* spp. This is because *T. asperellum* had a more aggressive growth in both YMA and PDA, but more so in PDA. *Rhizobium* spp. on the other hand has a restricted growth habit and thus it was difficult to establish the type of interaction. The alternative streaking with *T. asperellum* and *Rhizobium* spp. was not effective either in establishing their interaction because of the aggressive growth exhibited by *T. asperellum*. The

concentric rings of *Trichoderma* spp. changed to a green colour by the seventh day when the *T. asperellum* culture became fully mature and sporulated (Hafez *et al*., 2013).

The preliminary *in vitro* laboratory trials that were carried out in this research to determine the type of interaction between *T. asperellum* and *Rhizobium* spp. produced results that are in line with other research findings. The documented research proposes that *Trichoderma* spp. has antagonistic effects on most micro organisms (Paulitz and Belanger, 2001) and especially on plant pathogenic fungi such as *Fusarium oxysporium* and *Rhizoctonia solani* (Hafez *et al*., 2013). The inhibition of *Rhizobium spp*. by *Trichoderma* spp. was found to be between 49.7 % - 100 % which indicate that *Trichoderma* spp. was highly inhibitive on *Rhizobium spp*. The inhibitory effects of *T. asperellum* on *Rhizobium* spp. had suppressed the *Rhizobium* spp. culture so that by the third day, *Trichoderma* spp. had grown and almost covered half of the *Rhizobium* spp. culture. The inhibitory interaction observed between *T. asperellum* and *Rhizobium* spp. can be categorized into grade 4 according to the modified five types of interaction grades proposed for fungi interaction by Prince *et al*. (2011). Grade 4 represents sight inhibition of both the interacting organisms with narrow delineation line. *Trichoderma* spp. had a more aggressive growth and by the seventh day, the *Trichoderma* spp. had established on the whole plate and no traces of *Rhizobium* spp. were visible on the petri plate.

Interaction between *T. asperellum* and *Rhizobium* spp. in the pots also showed inhibition of nodulation by *T. asperellum*. However, most of the nodules were pink in colour showing that they were effective in fixing the nitrogen from the atmosphere into nitrates

(Somasegaran and Hoben, 1994). The numbers of nodules formed were highest in the control followed by the treatment with low level of *Trichoderma* spp. The treatment with high level *T. asperellum* had the least number of nodules formed. This is further indication that *Trichoderma* spp. actually inhibits nodulation in the French beans especially in high levels. This is possibly due to the fact that the treatment with *T. asperellum* inhibited the activity of *Rhizobium* spp. in the rhizosphere thus leading to a reduction in nodulation (Bandara, 1976). *Trichoderma asperellum* reduced the size and the number of nodules observed in the French beans that were grown in the greenhouse. When nodulation is compromised, the ability of the legumes, in this case the French beans, to carry out biological nitrogen fixation is reduced. This may lead to significant reduction in yields especially in nitrogen deficient soils where the plant does not have adequate supply of nitrates (HCDA, 2011).

Currently there is no documented research that has been carried out to show the effects of *Trichoderma* spp. on nodulation in any of the legumes. However, research has been carried out to show the antagonistic effects caused by other biological control agents on nodulation especially the effects of *Fusarium solani* on root nodule formation in *Phaseolus vulgaris* (Bandara, 1976). The research yielded similar results to those realized with interaction between *T. asperellum* and *Rhizobium* spp. in this research. The earlier research results of *Fusarium* spp. on nodulation suggested that the presence of *F. solani* in the soil rhizosphere reduced the number of root nodules. This could be probably due to interaction between the fungi and soil *Rhizobium* spp. rather than through direct effects on the bean plants (Bandara, 1976). The same case could also apply to the inoculation of

the French beans with *Rhizobium* spp. and treatment with *T. asperellum* since although the number of nodules reduced in the treatments with *T. asperellum*, the growth parameters investigated in the French beans were not affected.

As expected the number of nematodes in the control was very high compared to the other treatments with *T. asperellum* and Bionematon. *Trichoderma* spp. has been fronted as a potential biocontrol agent of root-knot nematodes in French beans (Kariuki *et al*., 2010). This significant reduction in the number of nematodes caused by the application of *Trichoderma* spp. is in accordance with research findings on parasitism of *Trichoderma* spp. on *M. javanica* and role of gelatinous matrix (Sharon *et al.,* 2007). The research by Sharon *et al.* (2007) showed that *T. asperellum* parasitizes on egg masses, their derived eggs and the second stage juveniles (J2). When the egg masses are destroyed, the number of the infective J2's is reduced as well as the overall number of nematodes. Thus this explains the reason for the reduction in the number of J2 nematodes that were observed from the soils treated with *T. asperellum* compared to the soil in the control. Treatment with high level of *T. asperellum* had the least number of nematodes indicating that *T. asperellum* was effective in controlling the nematodes.

The result on the number of nematodes reflects a similar trend as for the galling index. These results were as expected because the presence of nematodes influences the presence of root galls in the root system of the French bean plants. Thus the higher the nematodes number the higher the galling index and the less the number of nodules. This is because presence of nematodes reduces formation of nodules. Although the mean for

nodule number decreased in the treatments with *T. asperellum*, the mean for nodule biomass in different treatments was not significantly different. This is possibly due to the reduced size of the nodules that were formed in the control and at low level treatment with *T. asperellum* thus leading to decrease in biomass. Previous research has shown that the presence of nematodes reduced nodulation in the French beans as presented by Singh and Reddy (1981).

Although *T. asperellum* was found to inhibit nodulation and the growth of *Rhizobium* spp. *in vitro*, there was no significant effect of *T. asperellum* on the growth of the French bean plants. This was portrayed by the lack of differences in the growth parameters that were measured in the French beans. The results of this research indicated that there was no significant difference in the shoot biomass, root biomass as well as in root collar diameter from the French bean plants grown in the pots under different treatments. As suggested from the research carried out by Bandara (1976) on the effect of *Fusarium solani* on nodulation in *Phaseoli vulgaris*, the inhibition of *Rhizobium* spp. in the soil by the *T. asperellum* did not affect the growth of French beans.

5.2 Conclusion

i) Three *Rhizobium* spp. isolates were found to infect French beans in Mwea area, Kirinyaga County, in Kenya.

ii) *Trichoderma asperellum* inhibited the growth of *Rhizobium* spp. *in vitro*.

iii) *Trichoderma asperellum* also inhibited nodulation and reduced galling in the French beans grown in the greenhouse.

iv) Although application of *T. asperellum* inhibited nodulation in French beans, it had no effect on plant growth parameters which were; shoot biomass, root biomass and root collar diameter.

5.3 Recommendations

In light of this study, the following is recommended;

i) More *Rhizobium* spp. strains to be screened for resistance and tolerance to *Trichoderma* spp.

ii) More studies should be carried out on other *Trichoderma* spp. used as biological control agents.

iii) Molecular and biochemical studies to establish the mechanism behind the antagonism between *Trichoderma* spp. and *Rhizobium* spp. to be carried out.

iv) Since nodulation in French beans was found to reduce with application of *T. asperellum*, there is need to determine if nitrogen application is compromised and consequently if there is need to revise the amount of nitrogenous fertilizers applied to legume that have been treated with *T. asperellum*.

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